Universidade de Aveiro Departamento de Biologia
2010

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Sónia Alexandra Leite Velho Mendo, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro e Doutor Timothy Rutland Walsh, Professor Associado do Departamento de Microbiologia Médica da Universidade de Cardiff, Reino Unido e coordenação empresarial sob a orientação do Dr. Elmano José da Cruz Ramalheira, Assistente Graduado do Hospital Infante D. Pedro.

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“The route may be straightforward or somewhat circuitous, but beyond any doubt, the journey will be completed.”

Peter Bennett

o júri

presidente

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palavras-chave

Resistência a antibióticos, bactérias de Gram-negativo, IS*CR,* integrões de classe 1, genes *qnr*, β-lactamases.

resumo

A prevalência de bactérias resistentes a antibióticos em ambiente hospitalar tem vindo a tornar-se dramática e preocupante a nível mundial. Contudo, com a utilização inadequada de antibióticos em áreas tão diversas como a veterinária, a aquacultura e a agricultura, esta deixou de estar confinada ao ambiente hospitalar, sendo o ambiente um reservatório natural de microganismos resistentes a estes compostos.

O conhecimento detalhado dos determinantes de resistência a antibióticos presentes nestes ambientes, sejam estes genes de resistência ou estruturas envolvidas na sua mobilização, é fundamental, não só do ponto de vista do conhecimento como para a eventual implementação de medidas de contenção da sua disseminação. Neste contexto, são necessários estudos que permitam conhecer o panorama mais realista da distribuição destes determinantes de resistência a antibióticos, quer no meio ambiente quer no ambiente clínico.

Assim, constituiu objectivo principal deste trabalho contribuir para o conhecimento do panorama actual da prevalência e distribuição dos elementos IS*CR*, bem como de outros determinantes de resistência a antibióticos em espécies de Gram-negativo clinicamente relevantes (*Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii* e *Citrobacter freundii*) recolhidas a partir de amostras de pacientes do Hospital Infante D. Pedro, EPE, Aveiro, Portugal, entre 2006 e 2008. Adicionalmente, foram também recolhidas bactérias de Gram-negativo do meio ambiente, a partir de amostras de águas e de vísceras de peixes, nas quais foram igualmente pesquisados os elementos acima referidos. À excepção dos isolados de *E. coli* e de Gram-negativo ambientais, todas as estirpes estudadas foram seleccionadas com base no seu perfil de multirresistência a antibióticos.

Os resultados mostraram que em todas as espécies recolhidas no ambiente hospitalar foi detectada a presença de elementos IS*CR*, do tipo IS*CR1* ou IS*CR2*. O elemento IS*CR1*, foi encontrado em isolados de *E. coli, K. pneumoniae* e *C. freundii* e o elemento IS*CR2* em isolados de *A. baumannii*. Não foi detectada a presença de IS*CR*s nos isolados de Gram-negativo ambientais, o que sugere que a ocorrência dos mesmos é fortemente influenciada pela pressão selectiva exercida pelo ambiente em que os microrganismos se encontram.

Genes *qnr* e integrões de classe 1 foram os determinantes de resistência mais frequentemente encontrados associados aos elementos IS*CR1*. Os vários determinantes de resistência foram encontrados em diferentes contextos genéticos e localizados em estruturas móveis, nomeadamente em plasmídeos.

O elemento IS*CR2* presente em isolados de *A. baumannii* encontra-se associado ao gene *sul2* em todos os isolados, dentro de um mesmo contexto genético e com uma localização cromossomal. Contudo, o contexto genético encontrado nestes isolados é novo não tendo sido descrito até à data em outros microrganismos.

O presente estudo constitui a primeira descrição de elementos IS*CR* intrinsecamente ligados a genes de resistência a antibióticos, em Portugal. Uma vez que estes elementos parecem ser responsáveis pela mobilização de um grande número de genes de resistência a antibióticos, a sua elevada incidência entre estirpes resistentes e multirresistentes, bem como a sua associação com genes de resistência é preocupante e requer vigilância.

keywords

Antibiotic resistance, Gram-negative bacteria, IS*CR*, class 1 integron, *qnr* genes, β-lactamase genes.

abstract

Within the hospital environment, antibiotic resistant bacteria are a problem of concern worldwide. However, the antibiotic resistance has breached outside the confined hospital environment due to an inappropriate and intensive use of antibiotics in areas such as veterinary, aquaculture and agriculture, being the natural environment also a reservoir of antibiotic resistant microorganisms. Accurate and detailed studies focusing on the existing resistance determinants, either resistance genes or structures enhancing/involved in their dissemination, is needed in order to provide a realistic scenario of their distribution as well as for the implementation of measures that could prevent the dissemination of those genes. Thus, studies giving a wider and realistic panorama of the distribution of antibiotic resistance genes in both the natural and the hospital environment are of utmost importance.

The main goal of this thesis was to contribute to the present knowledge of distribution and prevalence of antibiotic resistance genes and IS*CR*s elements in clinically relevant Gram-negative species (*Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii* and *Citrobacter freundii*) collected from clinical specimens from patients in the Hospital Infante D. Pedro, EPE, Aveiro, Portugal between 2006 and 2008. Additionally, environmental Gram-negative isolates, collected from water and fish guts were also screened for the presence of the ISCR elements. All the isolates that were included in the present study were multi-drug resistant, with the exception of *Escherichia coli* and the Gram-negative environmental isolates.

The results obtained revealed that the IS*CR1 and ISCR2* elements were present in all the species collected within the hospital settings. IS*CR1* was present in *E. coli*, *K. pneumoniae* and *C. freundii* isolates and IS*CR*2 was detected in the *A. baumannii* isolates. The presence of IS*CR*s was not detected among the environmental Gram-negative isolates, suggesting that the occurrence of IS*CR* is clearly biased by the pressure exerted by the environment from where the microorganisms were isolated. *qnr* genes and class 1 integrons were the main resistance determinants found associated with IS*CR1* and were both described in different genetic contexts, located on mobile structures such as plasmids. Moreover, the IS*CR2* element was found associated with the *sul*2 gene in all the *A. baumannii* isolates with the same genetic context and located on the chromosome. Noteworthy is that the genetic context described is unique and was never described before in other microorganisms. This is the first study reporting the presence of IS*CR* elements in Portuguese isolates. Since these elements are responsible for the mobilization of several antibiotic resistance genes and were found intrinsically linked to them, their high incidence, among multi-drug resistant isolates is disconcerting and requires surveillance.

List of original publications

This thesis includes results from the following publications:

Ferreira S., Paradela A., Toleman M. A., Ramalheira E., Walsh T. R., Mendo S. (2010) Occurrence of IS*CR1*, class 1 integrons and antibiotic resistance genes in non-biased clinical *Escherichia coli* collected from day care, emergency room, and long term inpatients in Portugal. *Submitted to Antimicrobial Agents and Chemotherapy* – (**Chapter 3**).

Ferreira S., Toleman M., Ramalheira E., da Silva G.J., Walsh T., Mendo S. (2010) First description of *Klebsiella pneumoniae* clinical isolates carrying both *qnr*A and *qnr*B genes in Portugal. *International Journal of Antimicrobial Agents*. 35 (6) pp. 584-86 – (**Chapter 4**).

Ferreira S., Paradela A., Velez J., Ramalheira E., Walsh T., Mendo S. (2010) Carriage of *qnr*A1 and *qnr*B2, $bla_{\text{CTX-M-15}}$, and complex class 1 integron in a clinical multiresistant *Citrobacter freundii* isolate. *Diagnostic Microbiology and Infectious Diseases.* 67 (2) pp.188-90 – (**Chapter 5**).

Ferreira S., Li H-Y., Toleman M., Mendo S., Walsh T. R. (2010) Association of IS*CR2* with antibiotic resistance genes from Latin American and Portuguese *Acinetobacter baumannii* isolates. *Submitted Antimicrobial Agents and Chemotherapy* – (**Chapter 6**).

List of Tables

List of Figures

List of Abbreviations

3'-CS: Conserved segment 3'

5'-CS: Conserved segment 5'

59-be: 59 base element

AES: Advance expert system

Bp: Bases of pairs

CIA: chloroform/isoamyl alcohol

CS: Conserved segment

CLSI: Clinical and Laboratory Standards Institute

CR: Common region

DC: Day Care

Dhps: Dihydropteroate synthase

DNA: Deoxyribonucleic acid

EMBL: European molecular biology laboratoty

ER: Emergency room

ESBL: Extended-spectrum β-lactamase

HGT: Horizontal gene transfer

IP: Inpatients

IR: Inverted repeats

IS: Insertion sequence

IS*CR*: Insertion sequence common region

Kb: Kilobases

MBL: Metallo-β-lactamases

MDR: Multi-drug resistant

MGE: Mobile genetic element

MIC: Minimal inhibitory concentration

NAG: N-acetylglucosaminase

NAM: N-acetylmuramic acid

ORF: Open reading frame

PBP's: Penicillin Binding Proteins

PCR: Polymerase chain reaction

PFGE: Pulsed-field gel electrophoresis

PMQR: Plasmid mediated quinolone resistance

QRDR: Quinolone resistance determining region

RC: Rolling-circle

RI: Resistance integrons

RNA: Ribonucleic acid

Tn: Transposon

VGT: Vertical gene transfer

VR: Variable region

1. Introduction

1. Introduction

In the beginning of the last century, several pharmaceutical companies made substantial efforts on the research and development of new antibiotics (Alekshun and Levy 2007). The current era of antimicrobial chemotherapy began in 1935, with the discovery of sulphonamides. Later in 1940, it was demonstrated that penicillin could be an effective therapeutic substance. Since the introduction of penicillin in the 1940s, many antibiotics have been approved and launched into the market. Their availability transformed clinical practice, in a way where bacterial infections that were the scourge of humanity for so long, have until recently been overlooked, rather than being considered as life-threatening conditions (Bennett 2008; Hawkey 2008). Nonetheless, the discovery and development of a multitude of antibiotics have undoubtedly contributed to a decrease in human mortality associated with bacterial infections prior to the "antibiotic era" (Bennett 2008; Harbottle *et al.* 2006). Although Alexander Fleming selected and described mutants resistant to penicillin soon after he discovered the antibiotic, thereby foreseeing resistance to antibiotics, no one could predict how quickly bacteria would acquire the capacity to deal with multiple antibacterial agents (Alekshun and Levy 2007; Harbottle *et al.* 2006; Hawkey 2008). Indeed, bacteria exhibited different mechanisms of resistance, immediately after antibacterial drugs were deployed (Tenover 2006). Strains of *Shigella* bearing self-replicating and self-transferrable plasmids were easily selected and propagated during a period of considerable sulfonamide use in Japan after World War II (Watanabe 1963). Antibiotics such as streptomycin, chloramphenicol, and tetracycline were subsequently introduced in the treatment of the sulphonamide-resistant organisms. Nonetheless, *Shigella* and *Escherichia coli* strains bearing resistance to the aforementioned agents were identified in 1955 (Alekshun and Levy 2007).
The rapid acquisition and dissemination of resistance in few years were just the first signs of what was to come and led us to a point where at present, all known antibiotics currently available for clinical use in human and veterinary medicine have a resistance mechanisms reported (Bennett 2008; Harbottle *et al.* 2006).

Moreover, the introduction of new classes or modifications of older classes of antimicrobials over the past six decades has been matched, slowly but surely, by the systematic development of new bacterial resistance mechanisms (Harbottle *et al.* 2006). We tend to forget that bacteria have inhabited the planet for approximately three and a half billion years, somewhat longer than mankind, and that they had to adapt on innumerable occasions to toxic substances suddenly introduced into their environments (Bennett 2008) (Figure 1).

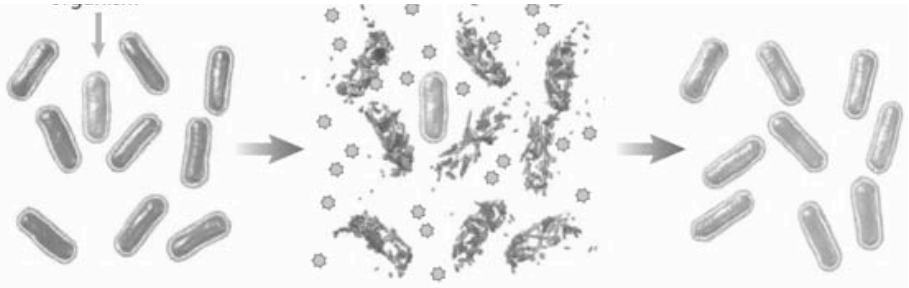


Figure 1 – Effect of the selective antibiotic pressure in bacteria (Mulvey and Simor 2009).

Obviously, not all of the changes that allow this adaptation, for instance antibiotic resistance, are acquired by design (Bennett 2008; Harbottle *et al.* 2006; Walsh 2006). The vast majority of these changes makes no improvement on the survival of an individual and are eliminated from the population over time, by a range of proofreading and DNA repair mechanisms (Boerlin and Reid-Smith 2008). However, those changes that confer advantage are conserved and, given appropriate circumstances, may undergo clonal amplification (Bennett 2008).

Most likely, resistance genes and mechanisms allowing for their subsequent spread existed long before the discovery and introduction of modern therapeutic antimicrobials. For instance, antimicrobial resistant bacteria estimated to be over 2000 years old have been recovered from glacial samples obtained from the Canadian Arctic Archipelago, while a more recent study detected TEM-type β-lactamases from a metagenomic library of cold-seep sediments of deep-sea Edison seamount (near Papua New Guinea) estimated to be about 10,000 years old (Harbottle *et al.* 2006).

Evidence such as the emergence of organisms resistant to several antibiotics, including purely synthetic agents like quinolones, suggests not only that our surroundings can act as a reservoir for new resistance mechanisms, but also that the drugs used to treat infectious diseases have extended half-lifes outside of the hospital environment. In addition, many antimicrobial molecules have existed for millennia within the environment, selecting and promoting growth of resistant strains (Alekshun and Levy 2007).

Over the years, the continued use of antibiotics generated an enormous selective pressure leading to organisms bearing additional resistance, and thus multi-drug resistance through changes in penicillin-binding proteins (PBPs), drug modification, drug destruction, mutated drug targets, enhanced efflux pump expression and altered membrane permeability (Figure 2) (Alekshun and Levy 2007).

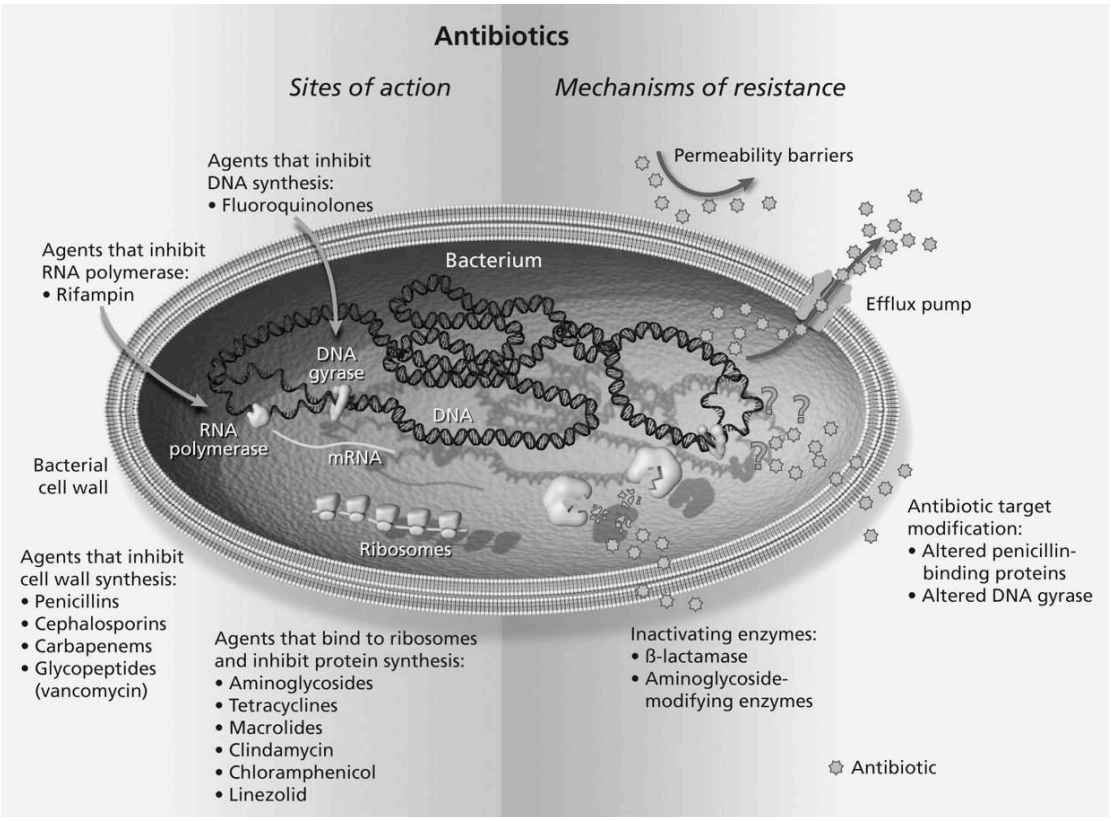


Figure 2 – Antibiotics sites of action and microbial mechanisms of resistance (Mulvey and Simor 2009).

In fact, resistance to both natural antimicrobial agents and synthetic derivates, as well as completely synthetic antimicrobials has been observed in soil-dwelling actinomycetes, with some, displaying resistance mechanisms not traditionally encountered in bacterial pathogens isolated from clinical environments (Harbottle *et al.* 2006). Surprisingly, researchers found every tested isolate to display resistance to at least six to eight different antimicrobial agents, and in some cases, as many as twenty (Harbottle *et al.* 2006). There appears to be a vast, yet broadly unknown reservoir of antimicrobial resistance genes lurking in natural environments, which may provide transferrable resources for other bacteria (Boerlin and Reid-Smith 2008).

Moreover, the continuous use of antibiotics contributes to selective pressures that may ensure survival of a particular subset of genetic rearrangements; namely, those involving genes conferring antibiotic resistance. Whether these rearrangements occur in bacteria able to cause infection is, arguably immaterial. Once it is established, it can then amplify the selected rearrangement, and contribute for its dissemination under appropriate conditions (Bennett 2008). When these conditions are established, the selected rearrangement will subsequently be spread horizontally by plasmids to other bacteria, including human pathogens (Bennett 2008).

1.2. Antibiotics

Although the boom of discovery and consequent use of antibiotics has happened in the beginning of last century, drugs have been used to the treatment of infectious diseases since the 17th century. However chemotherapy as a science began with Paul Ehrlich in the first decade of the 20th century, when he used an arsenic compound, salvarsan, to treat syphilis.

Antibiotics can be defined as any compound either synthetic or naturally produced by fungi, plant or bacteria, which in a small quantity, can inhibit the growth of microorganisms or even kill them. The antibacterial activity of an antibiotic can be measured *in vitro* by determining the minimal inhibitory concentration (MIC). While some antibiotics merely inhibit the multiplication of bacteria and are therefore called bacteriostactic, others actively kill microorganisms thus having bactericidal activity.

Antibiotics can be classified according to their chemical structure, biological origin and therapeutic use. In addition, most antimicrobial agents used for the treatment of bacterial infections may be categorised according to their principal mechanism of action (Tenover 2006). There are five general areas of bacterial metabolism that are targets for the development of new antimicrobials: cell wall synthesis, protein synthesis, RNA synthesis, DNA synthesis and intermediary metabolism (Table 1) (Hooper 2001).

Antibiotics can also be classified according to their spectrum of action. Therefore, narrow-spectrum antibiotics act selectively against pathogens while broad-spectrum antibiotics are effective against a wide-range of pathogens.

Table 1 – Antibiotics and their respective mechanisms of action (adapted from http://www.wiley.com).

Antibiotic	Mechanism
Inhibitors of cell wall synthesis	
Carbanicillin	Inhibits transpeptidation enzymes. Activates lytic enzymes of cell wall.
Penincillin	Inhibits transpeptidation enzymes. Activates lytic enzymes of cell wall. The affected bacterium will eventually lyse because the unsupported cell wall cannot withstand its growth.
Vancomycin	Inhibits transpeptidation in cross-linking peptidoglycans, interferes with bacterial cells at many levels, disrupting cell wall synthesis, interfering with RNA, and damaging the plasma membrane.
Inhibitors of nucleic acid synthesis	
Ciprofloxacin	Inhibits DNA gyrase; Interferes with DNA replication.
Rifampicin	Blocks RNA synthesis by binding and inhibiting RNA polymerase.
Inhibitors of protein synthesis	
Chloramphenicol	Blocks formation of new peptide bonds during protein synthesis by binding to the 50S subunit of the ribosome.
Erithromycin	Binds the 50S subunit and blocks translocation of the new protein on the ribosome, thus effectively halting synthesis.
Fusidic acid	Blocks translocation.
Linezolid	Binds rRNA to prevent translation initiation and thus protein synthesis.
Streptomycin	Binds the 30S ribosomal subunit of the bacteria and prevents the ribosome from forming the complex necessary to initiate protein translation.
Tetracyclines	Binds to the 30S subunit and blocks the addition of the amino acids, producing incomplete and probably non-functional proteins.
Metabolic inhibitors	
Dapsone	Interferes with synthesis of the folic acid, which is required for the synthesis of purines and thymidine and for the synthesis of the amino acids methionine and and glycine.
Sulfonamides	Competitively inhibits dihydropteroate synthase. These drugs can also be incorporated into a compound that resembles dihydrofolate and that in turn can inhibit another enzyme in the pathway, dihydrofate reductase.
Trimethoprim	Inhibits dihydrofolate reductase, blocking tetrahydrofolate synthesis.

1.2.1 β-lactams

β-lactam antibiotics represent the most diverse class of antibiotics that are widely used due to their spectrum of action, low toxicity and pharmacokinetic characteristics. There are six different groups (Figure 3) into which they can be divided: penicillins, cephalosporins, carbapenems, cephamycins, monobactams and β-lactamase inhibitors (Smet *et al.* 2008).

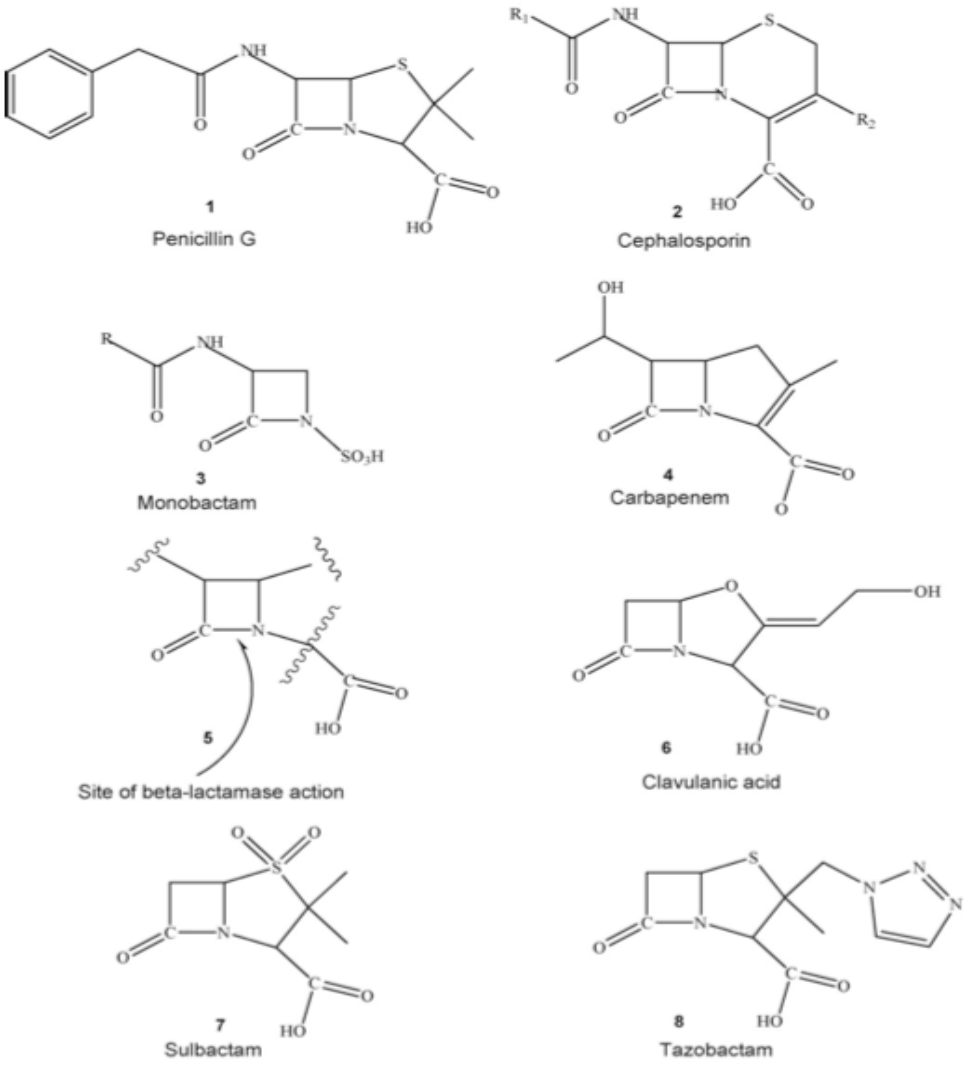


Figure 3 – β-lactams structure. Five classes are represented: 1) penicillins 2) cephalosporins, 3) monobactams 4) carbapenems, and 6,7,8) β-lactamase inhibitors (clavulanic acid, sulbactam and tazobactam) (Smet *et al.* 2008).

β-lactam antibiotics are bactericidal and are known to interact directly with PBPs forming covalent linkages, inhibiting the synthesis of peptidoglycan cross-linking, the main constituent of the cell wall. The structural profile and chemical composition of cell walls are very similar between Gram-negative and Gram-positive bacteria. However, the main difference is related to the structural component that ensures the mechanical strength of the cell wall – the peptidoglycan (or murein). This heteropolymer consists of linear chains of two amino sugars, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), evidenced in figure 4, arranged alternately and joined by glicosydic bonds.

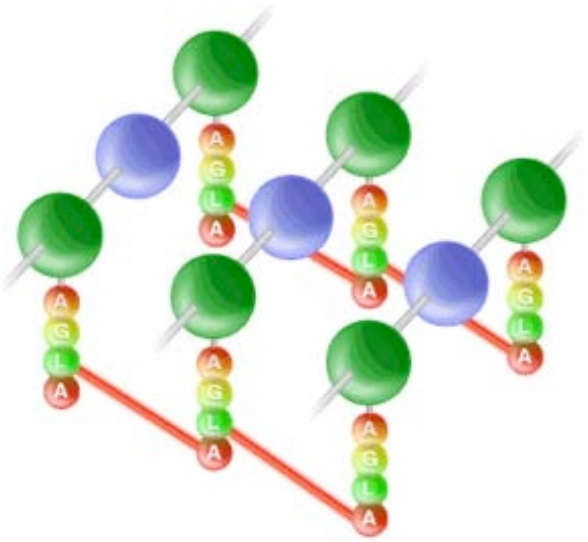


Figure 4 – A schematic representation of the peptidoglycan structure. The NAM and NAG sugars are shown as green and blue spheres respectively. The tetrapeptides linked to NAM are cross-linked by a pentaglycine peptide, shown as red lines linking the D-glutamine (L) to the D-alanine (A) (http://www.scq.ubc.ca/prescription-antibiotics-how-exactly-do-these-drugs-work/).

β-lactams as transpeptidase inhibitors block the conversion of immature to mature peptidoglycan, thereby inhibiting enlargement of the bacterial cell wall (Hooper 2001). They are preferred not only because of their efficacy and safety, but also because their activity can be extended or restored by chemical manipulation. No other antibiotic class has such chemical malleability and versatility (Livermore and Woodford 2006). Inevitably, however, their heavy usage had devastating

consequences as it ultimately selected for resistance. The first penicillin-resistant *Staphylococcus aureus* was identified in the mid-1940s and expressed a β-lactamase that afforded resistance to penicillin's (Alekshun and Levy 2007; Hawkey 2008). With the development of semi-synthetic penicillins (e.g. ampicillin and carbenicillin) in the early 1960s, the problem of resistance was overcome or, at least, postponed. However, their application was soon compromised by the spread of plasmid-mediated penicillinases (notably TEM-1) among Enterobacteriaceae (Abraham and Chain 1940).

A major breakthrough was achieved against β-lactamase mediated bacterial resistance, with the introduction into clinical practice of the third-generation extended-spectrum cephalosporins (e.g. cefotaxime and ceftazidime). In the early 1980s, when cephalosporins were introduced, virtually all Enterobacteriaceae were susceptible (Hawkey 2008; Livermore and Woodford 2006; Paterson and Bonomo 2005). Third-generation cephalosporins were strongly effective against most β-lactamase-producing organisms and had also the major advantage of narrowed nephrotoxic effects compared to aminoglycosides and polymyxins (Paterson and Bonomo 2005). These properties were fully explored in the subsequent 25 years, and have become antibiotics of choice worldwide. Once again, clinical use and/ misuse has selected for resistance and this cephalosporin resistance, along with dramatically rising enterobacterial resistance to fluoroquinolones, is now driving the use of carbapenems. Unfortunately, carbapenem resistance will not take long to emerge. In fact the use of carbapenems is very much under threat, especially in *P. aeruginosa* (Livermore and Woodford 2006).

β-lactamases are the main cause of resistance to β-lactam antibiotics in Gram-negative bacteria, and are increasing among clinical isolates, therefore compromising β-lactam usage. These enzymes are either chromosomally or plasmid and transposon mediated, and inactivate β-lactams by hydrolyzing the four-membered β-lactam ring, rendering the compound inactive (Smet *et al.* 2008).

1.2.1.1 β-lactamases

Antibacterial drugs can be altered by enzymes which can be divided into general classes: those such as β-lactamases that degrade antibiotics, and others (including the macrolide and aminoglycoside-modifying proteins) that perform chemical alterations (Alekshun and Levy 2007). Given the great diversity of enzymes produced, β-lactamases are commonly classified according to either molecular or functional properties. Based on these two properties two general schemes of classification were proposed: the Ambler molecular classification scheme and the Bush-Jacoby-Medeiros functional classification system (Bush *et al.* 1995; Queenan and Bush 2007).

The Ambler scheme divides β-lactamases into four major classes (class A, B, C, and D), relying upon the basis of their primary structure and not on phenotypic characteristics, despite lacking the detail concerning the enzymatic activity of the enzyme. In this scheme, classes A, C and D include β-lactamases with serine on their active site, whereas class B enzymes are metallo-β-lactamases with zinc on their active-site (Paterson and Bonomo 2005; Queenan and Bush 2007).

Isolation of the protein and determination of its isoelectric point, followed by enzymatic studies to determine substrate hydrolysis and inhibition characteristics, were the biochemical procedures used in the analysis of a new β-lactamase (Paterson and Bonomo 2005; Queenan and Bush 2007). Therefore, the relative hydrolysis rates of a broad spectrum of β-lactam substrates, and inhibitor profiles, allowed for the classification of a new β-lactamase. This functional classification scheme, proposed by Bush-Jacoby-Medeiros, evolved over many years into a widely accepted scheme currently dividing the known β-lactamases into four major functional groups (groups 1 to 4). Group 2 has multiple subgroups that are differentiated according to group-specific substrate or inhibitor profiles (Paterson and Bonomo 2005; Queenan and Bush 2007).

In particular, extended spectrum β-lactamases (ESBLs) of Bush-Jacoby-Medeiros group 2be, capable of conferring resistance to the penicillins, first-, second- and third-generation cephalosporins and aztreonam, that are also inhibited by clavulanic acid, have been thoroughly examined (Paterson and Bonomo 2005). The term ESBL is now under considerable debate and new classification criteria have been proposed to accommodate ESBLs with carbapenemase activity as well as class C and class D enzymes (Giske *et al.* 2009). ESBLs were first described in the mid-1980s. Earlier examples were derived from TEM-1, TEM-2 or SHV-1 differing from their ancestors by as few as one amino acid. This substitution resulted in a profound change in the enzymatic activity of the ESBLs, enlarging their hydrolytic capacity, so that they can now hydrolyze the third-generation cephalosporins or aztreonam (Livermore and Woodford 2006; Paterson and Bonomo 2005). As ESBLs are inhibited by clavulanic acid they can be differentiated from the AmpC-type β-lactamases (group 1) produced by organisms such as *Enterobacter cloacae* which have third-generation cephalosporins as their substrates, but are not inhibited by clavulanic acid (Paterson and Bonomo 2005). If a plasmid carries both an AmpC gene and an ESBL gene, the detection of the latter can be difficult; this requires the use of AmpC-stable cephalosporins, such as cefepime or cefpirome with clavulanate or boronic acid as inhibitors, although detection is not 100% reliable even when using these combinations (Hawkey 2008; Livermore and Woodford 2006).

1.2.1.1.1 SHV β-lactamases

SHV refers to *s*ul*h*ydryl *v*ariable. The inhibition of SHV activity by *p*-chloromercuribenzoate was thought to be substrate-related, and variable according to the substrate used for the assay, thereby the term SHV (Paterson and Bonomo 2005).

In 1983, a *Klebsiella ozaenae* isolate was discovered in Germany, that possessed a β-lactamase which efficiently hydrolyzed cefotaxime and to a lesser extent ceftazidime (Paterson and Bonomo 2005). This β-lactamase, designated SHV-2,

differed from SHV-1 by the replacement of glycine by serine at the position 238, and this mutation alone accounts for its extended-spectrum properties. Enterobacteriaceae outbreaks associated with SHV-type ESBLs have been reported, and have also been detected in *P. aeruginosa* and *Acinetobacter* spp. (Paterson and Bonomo 2005).

1.2.1.1.2 TEM β-lactamases

More than 170 TEM-type β-lactamases have been described, the majority of them are ESBLs. The TEM-type ESBLs are derivates of TEM-1 and TEM-2, and the amino acid changes in comparison with TEM-1 and TEM-2 are well documented at http://www.lahey.org/studies/temtable.html (Paterson and Bonomo 2005) . TEM-1 was first reported in 1965 from an *E. coli* isolate from a patient in Athens, Greece, named Timoneira (hence the designation TEM) (Paterson and Bonomo 2005). TEM-1 enzyme is now the commonest β-lactamase found in Enterobacteriaceae. The *bla* gene encoding the TEM-1 β-lactamase is the most frequently found Amp^R marker used in molecular biology (pBR and pUC plasmids). TEM-1 is a widespread plasmidic β-lactamase that attacks narrow-spectrum cephalosporins, cefamandole, and cefoperazone and all the anti-Gram-negative bacterium penicillins except temocillin. In 1987, a plasmid harbouring a β-lactamase, at the time termed CTX-1 based on its enhanced activity against cefotaxime, was detected in *Klebsiella pneumoniae* isolates collected as early as 1984, in France (Paterson and Bonomo 2005). Today, CTX-1 is termed TEM-3, and differs from TEM-2 by two amino acid substitutions (Paterson and Bonomo 2005).

1.2.1.1.3 CTX-M β-lactamases

Recently, a surprisingly fast disseminated and vast group of extended-spectrum β-lactamases has gained the attention of microbiologists worldwide (Rodriguez *et al.* 2004). These β-lactamases are named CTX-M enzymes with

CTX reflecting the potent hydrolytic activity against cefotaxime. This family of plasmid-mediated ESBLs has been classified in the Ambler class A and in group 2be of the Bush, Jacoby and Medeiros classification (Eckert *et al.* 2006). Organisms producing CTX-M-type β-lactamases typically have cefotaxime MICs in the resistance range (>64 μg/mL), while ceftazidime MICs values are usually in the apparently susceptible range (2 to 8 μg/mL) (Livermore and Woodford 2006; Paterson and Bonomo 2005). However, some CTX-M-type ESBLs can hydrolyze ceftazidime resulting in resistance. These enzymes hydrolyze cefepime with high efficiency, and contribute to cefepime MICs higher than those observed in bacteria producing other ESBLs types (Eckert *et al.* 2006; Paterson and Bonomo 2005).

CTX-M enzymes include more than sixty variants that are distributed into five different clusters (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25) according to their amino-acid sequence (Eckert *et al.* 2006; Su *et al.* 2008). Figure 5 shows a world distribution of the majority of the aforementioned clusters.

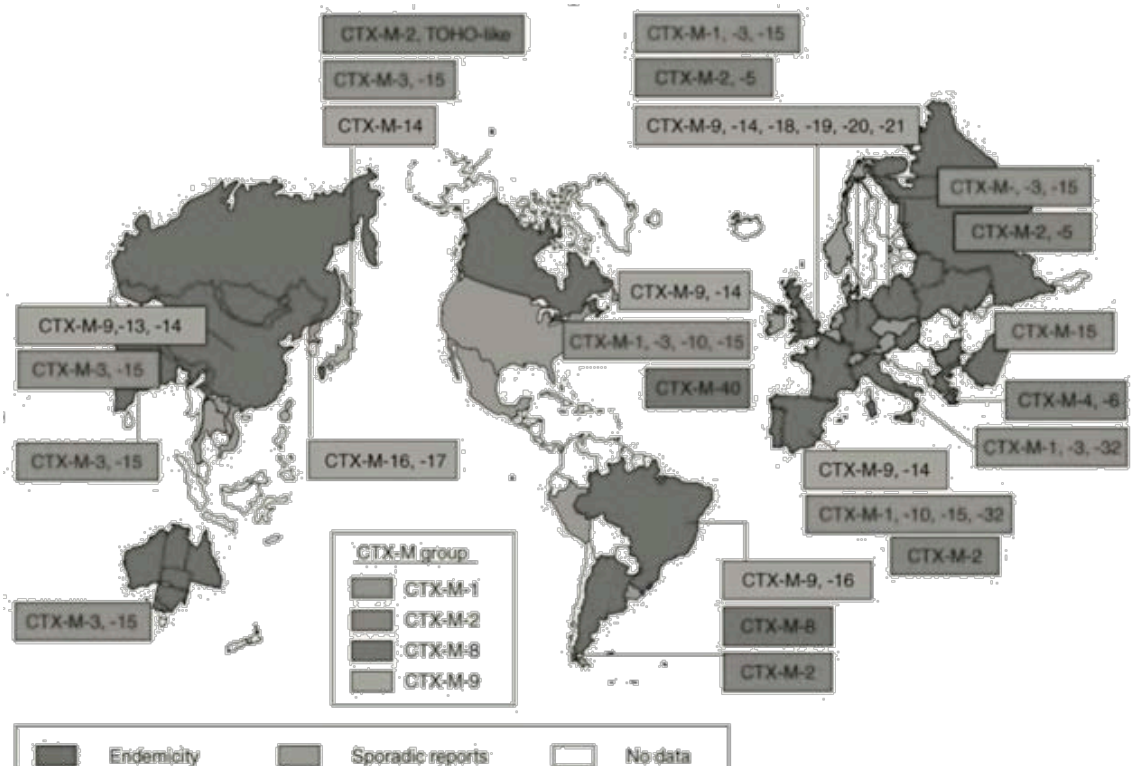


Figure 5 – Global distribution of the different CTX-M enzymes clusters (Cantón and Coque 2006).

Several reports have shown that *Kluyvera* spp., although a genus of little clinical relevance, carried the probable ancestor CTX-M enzymes. These enzymes evolved from chromosomal β-lactamase genes from *Kluyvera* spp., which the mobilization was facilitated by the insertion sequence IS*Ecp1* or related insertion sequences (Livermore and Woodford 2006; Paterson and Bonomo 2005; Rodriguez *et al.* 2004). Thus, it is safe to conclude that the rapid dissemination of $bla_{CTX\text{-}M}$ genes involves plasmid or strain epidemics, as well as mobile genetic elements (Bae *et al.* 2008).

1.2.2 Quinolones

As mentioned before, antibiotics can be isolated and synthesized from plant, fungi and bacteria. However unlike some of the first antibiotics discovered during the past century, quinolones were not isolated from living organisms but they are rather synthesized by chemists (Andriole 2005). Nalidixic acid (Figure 6) was the first compound to be produced in 1962, when it was accidently discovered as a by-product of the synthesis of chloroquine, an antimalarial compound (Andriole 2005).

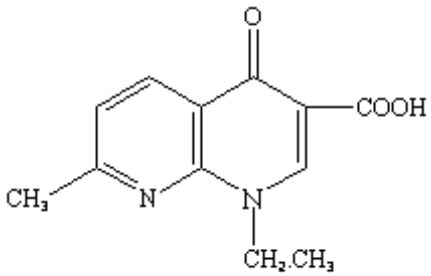


Figure 6 – Structure of nalidixic acid molecule (http://www.bmb.leeds.ac.uk/mbiology dna.html).

After the discovery of nalidixic acid, quinoles undergone several modifications to enhance their performance, creating analogous antibacterial agents. In fact, a key finding in the evolution of quinolones was the modification of the quinolone nucleus through the addition of different substituents at the N-1 (cyclopropyl group), C-6 (fluorine atom), C-7 (piperazine group) and C-8 (fluorine or a chlorine) (Andriole 2005).

Quinolones and fluoroquinolones are bactericidal antibiotics, commonly prescribed, that have been widely used in medicine due to their broad efficacy, immunomodulatory activities and also because they can be administrated orally (Kim *et al.* 2009a; Toleman *et al.* 2006b). They act against DNA metabolism inhibiting DNA synthesis, and at higher concentrations, RNA synthesis as well. Their primary targets are bacterial enzymes DNA gyrase and topoisomerase IV (Figure 7). The ability of quinolones to interact with these two targets within the bacterial cell, by stabilizing complexes of DNA and Type II and IV topoisomerases, mediated the effects on DNA metabolism (Andriole 2005).

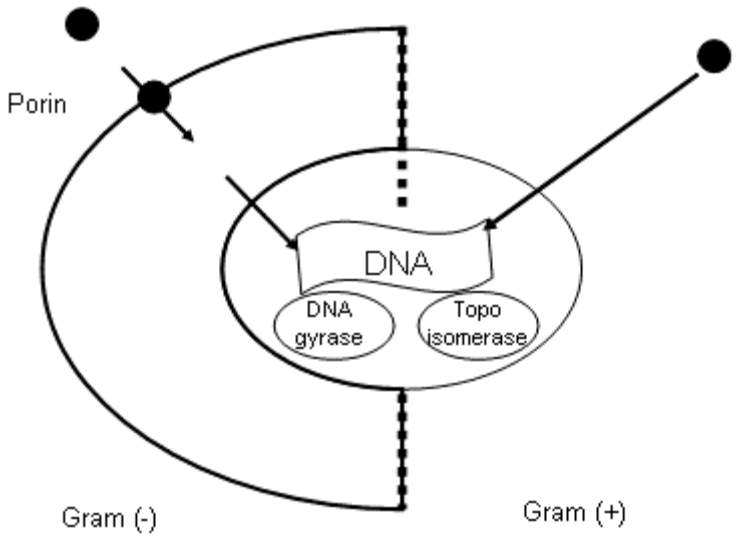


Figure 7 – Primary targets of quinolones in Gram-negative and Gram-positive bacteria (http://www.sbimc.org).

However, under new selection pressures, bacterial populations may respond with new mutations. Such mutations result in bacteria selection, in which new advantageous characteristics, are more prone to emerge in mutator than in non-mutator strains (Boerlin and Reid-Smith 2008). This mechanism is thought to play a role in the emergence of mutations leading to resistance to the antimicrobial agents, such as fluoroquinolones (Boerlin and Reid-Smith 2008). These mutations can gradually accumulate in most bacteria, mediating high-level fluoroquinolone resistance. Point mutations in the genes encoding DNA gyrase sub-units can alter the binding efficiency of quinolones, thereby reducing their efficacy or even preventing it (Kim *et al.* 2009b). Multiple mutations in the quinolone resistance determining region (QRDR) of genes such as *gyr*A, *gyr*B, and *par*C, cause an increase in the MICs values, and consequently decreased susceptibility to quinolone antimicrobials (Harbottle *et al.* 2006). Furthermore, in Enterobacteriaceae, topoisomerase based resistance to quinolones occurs generally in a two- step mutation process with moderate levels of resistance. The first mutation in the QRDR of the primary target, *gyr*A gives full resistance to narrow-spectrum quinolones, such as nalidixic acid, and decreased susceptibility to fluoroquinolones. Once established, higher levels of resistance may occur from additional mutations in one of the *gyr* or *par* genes, giving full resistance to fluoroquinolones (Nordmann and Poirel 2005; Smet *et al.* 2008). However, the primary drug target enzyme as defined by first-step quinolone resistance mutations often differs between Gram-positive and Gram-negative bacteria (Hooper 2001).

In compounds that are isolated from living organisms, sooner or later, mechanisms of resistance are expected to arise. However, as fluoroquinolones are, fully synthetic, naturally evolving antimicrobial resistance genes have not been considered a threat. Moreover, when quinolones were discovered, it was thought that this problem would take many years to occur since the bacteria exposed to these compounds would not have the ability to survive in their presence. Once again, the bacteria ability to adapt was underestimated.

A recently discovered example of enzymatic alteration of a synthetic antimicrobial involves the plasmid-borne variant of the original aminoglycoside *6´-N*-acetyltransferase, (AAC(6')-Ib-cr) that acetylates fluoroquinolones and reduces their activity (Harbottle *et al.* 2006; Kim *et al.* 2009b; Lascols *et al.* 2008; Robicsek *et al.* 2005b). Although the *aac(6')-Ib-cr* causes low-level resistance to ciprofloxacin, this gene was shown to act additively with another plasmid mediated quinolone resistance (PMQR) mechanism *qnr*A*,* and the mutant prevention concentration was significantly altered (Kim *et al.* 2009b; Robicsek *et al.* 2005b). Additionally, *qepA,* which encodes an efflux pump belonging to the major facilitator subfamily, was found in a plasmid, and also confers quinolone resistance (Perichon *et al.* 2007; Yamane *et al.* 2007).

Another example, already mentioned above, that has been reported worldwide is the plasmid-mediated quinolone resistance encoded by *qnr*A, *qnr*B, *qnr*C, *qnr*D or *qnr*S (Aihua *et al.* 2008; Cavaco *et al.* 2009; Jacoby *et al.* 2008; Nordmann and Poirel 2005). Qnr proteins are members of the pentapeptide family, that are able to protect topoisomerases reducing their susceptibility to fluoroquinolones and increasing the mutant selection window, ultimately increasing the selection of resistant mutations (Cavaco *et al.* 2007b; Nordmann and Poirel 2005). *qnr*A was the first *qnr* gene described in a *K. pneumoniae* in 1998 by Martinez *et al*, (Martínez-Martínez *et al.* 1998). It has subsequently been shown to be widely distributed and is always part of a complex *sul1*-type integron containing IS*CR1* (Park *et al*. 2007). *qnrS* was first described in *Shigella flexineri* in 2005 and *qnrB* in 2006 in *K. pneumoniae* and *E. coli* (Lascols *et al.* 2008). *qnrC* and *qnrD* were reported in *Proteus mirabilis* and in a *Salmonella enterica* strain respectively (Cavaco *et al.* 2009). Subsequent reports have shown that these genes are also present in other species of Enterobacteriaceae and have been found in many countries from Europe, Asia and the United States of America (Lascols *et al.* 2008; Robicsek *et al.* 2006). Transferrable *qnr* genes are usually carried by large conjugative plasmids (50 to 180kb) that often encode ESBLs or AmpC-type β-lactamases (Lascols *et al.* 2008).

Based on this strong association between *qnr* genes and plasmids carrying cephalosporinase genes, ceftazidime resistance is often an inclusion criterion for selection of strains carrying a *qnr* gene (Robicsek *et al.* 2006). The dissemination of plasmids carrying *qnr* genes can potentiate the rapid development of higher level of quinolone resistance in organisms currently classified as susceptible (Robicsek *et al.* 2005b). In fact, a recent study associates quinolone resistance in Enterobacteriaceae with an increasing prevalence and diversity of PMQR genes in isolates collected consecutively during a given timeframe (Kim *et al.* 2009a; Kim *et al.* 2009b).

Quinolones were a useful resource to fight MDR isolates, but still highly susceptible to fluoroquinolones. These isolates would have been expected to have a low likelihood for the emergence of quinolone resistance (Kim *et al.* 2009a).
The rapid emergence of PMQR, together with the increasing use of flouroquinolones, created the opportunity of highly quinolone-resistant clinical isolates to arise, reducing the already narrow window of therapeutic options.

1.2.3 Aminoglycosides

Aminoglycosides are a very heterogeneous group in their chemical composition, pharmacological properties and antibacterial properties. They are also among the most commonly used broad-spectrum antibiotics to treat infectious diseases caused by Gram-negative bacteria (Galimand *et al.* 2003; Kotra *et al.* 2000; Shakil *et al.* 2008; Toleman *et al.* 2006b). The aminoglycosides are very valuable as anti-infectives, since a vast majority exhibits bactericidal activity, have predictable pharmacokinetics, and often act in synergy with other antibiotics (Kotra *et al.* 2000; Shakil *et al.* 2008). Furthermore, unlike other classes of antibiotics, several aminoglycosides have been employed in clinical use for several decades (Kotra *et al.* 2000).

Agents belonging to this class of antibiotics act either at the level of bacterial protein synthesis by blocking the ribosome subunits irreversibly or by disrupting the integrity of bacterial cell membrane (Galimand *et al.* 2003; Shakil *et al.* 2008). Resistance, due to chromosomal mutations, is uncommon in this class of antibiotics even though the binding site for aminoglycosides is in the rRNA (Alekshun and Levy 2007; Kotra *et al.* 2000). Absence of alterations in the rRNA is predictable given the fact that its central function is protein biosynthesis and that this function is so well preserved across genera that it cannot be impaired by the possibility of such structural mutations (Kotra *et al.* 2000). However, aminoglycosides resistance does exist and has been widely reported, although not on the same scale as the resistance to β-lactam antibiotics (Kotra *et al.* 2000). Resistance to these compounds results from the action of a vast number of aminoglycoside-modifying enzymes specified by genes on transferable elements that are widely spread in clinically relevant organisms. Many of these enzymes are found on integrons and other mobile elements where they associate with additional resistance determinants (Alekshun and Levy 2007).

Aminoglycosides resistance occurs due to four major mechanisms: (a) the deactivation of aminoglycosides by proteins that N-acetylate (acetyltransferases), phosphorylate (phosphotransferases) and adenylate (nucleotidyltransferases) the compounds. The acetyltransferases are capable of modifying tobramycin, gentamicin, netilmicin and amikacin; the nucleotidyltransferases proteins alter the activity of tobramycin; and the phosphotransferases affect amikacin susceptibility (Alekshun and Levy 2007; Kotra *et al.* 2000). (b) the reduction of the intracellular concentration of aminoglycosides by changes in outer membrane permeability, decreased inner membrane transport, active efflux and drug trapping (Kotra *et al.* 2000; Shakil *et al.* 2008); (c) the alteration of the 30S ribosomal subunit target by mutation and (d) methylation of the aminoglycoside binding site (Shakil *et al.* 2008).

1.2.4 Sulfonamide and Trimethoprim

The discovery of sulfonamides occurs in 1932; however, it was only published in 1935. Sulfonamides were the first antimicrobials developed for large-scale production into clinical practice. Their discovery pales only in comparison with that of Fleming's chance to discover penicillin, as the initial antibacterial activity was not proven to be *in vivo* (Alekshun and Levy 2007; Tenover 2006). The drug had no effect at all in the test tube; however, in the living body it was found to act as a prodrug being released from the inactive dye as a small, colourless and active compound. Sulfonamides can be used in combination with another drug, trimethoprim, producing a synergistic antibacterial effect by inhibiting successive steps in the folate synthesis (Figure 8). The combination of the two drugs is known as co-trimoxazole.

Trimethoprim, introduced in 1968, inhibits dihydrofolate reductase and consequently inhibits synthesis of tetrahydrofolic acid, which is an essential precursor in the *de novo* synthesis of the intermediate thymidine monophoshpate (dTMP), precursor of the DNA metabolite thymidine triphosphate (dTTP).

Laboratory experiments using *E. coli*, *Streptococcus pneumoniae* and clinical isolates of *Campylobacter jejuni* and *Haemophilus influenzae* showed that mutations in the gene specifying dihydropteroate synthase decrease the enzyme's affinity for the sulfonamides (Alekshun and Levy 2007). Additionally, mutations in the chromosomal gene specifying dihydrofolate reductase can result in over-expression of an enzyme with reduced affinity for trimethoprim and thereby offer very high-level trimethoprim resistance in *E. coli* and *H. influenzae* (Alekshun and Levy 2007).

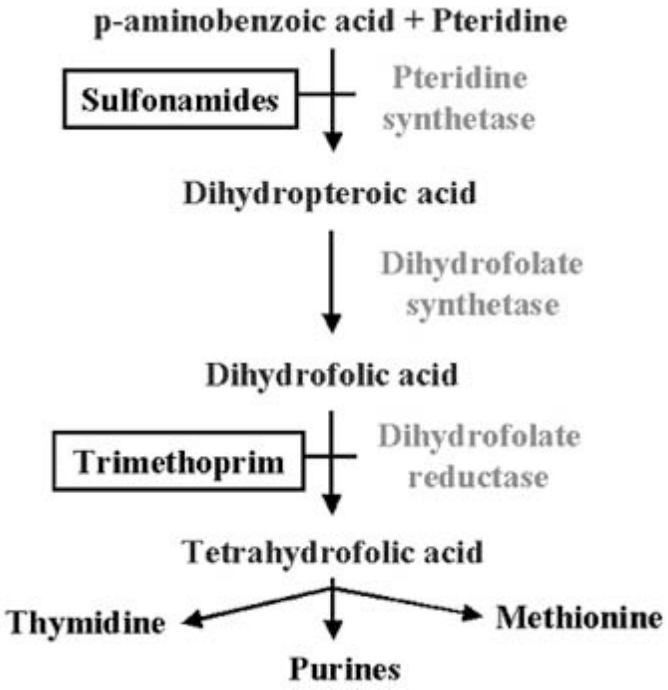


Figure 8 – Sites of inhibition of folic acid metabolism by sulfonamides and trimethoprim (http://pathmicro.med.sc.edu/mayer/antibiot.htm).

The synergetic effect of co-trimoxazole is also affected by the acquisition of genes specifying enzymes that are insensitive to drug inhibition. Among these genes, *sul1* and *sul2* are the main determinants of clinical resistance to sulfonamide, whereas *sul3* was found to be prevalent in farm animals (Alekshun and Levy 2007).

dfr genes are responsible for trimethoprim resistance and more that 20 variants have been described. *sul* genes are present on class 1 integrons (*sul1*) or plasmids (*sul2*) and associated with IS*CR*s, whereas *dfr* variants move from organism to organism on class 1 and 2 integrons. *dfr1* is the most common in Gram-negative bacteria and is present on the Tn*7* transposon, thereby permitting its integration into the *E. coli* chromosome (Alekshun and Levy 2007).

1.3 Bacterial resistance mechanisms

Bacteria can avoid potentially toxic compounds that threaten their survival using single or combined mechanisms of resistance involving different elements (Figure 9); all they need is time to adapt to the changes in their surroundings. Therefore, throughout history, there has been a continual battle between humans and the plethora of microorganisms causing infection and disease (Tenover 2006).

1.3.1 Intrinsic resistance

The mechanisms used to attain resistance can be either intrinsic or acquired. Usually resistance due to the former can be described as a natural phenomenon displayed by all members of a species, and is a function of physiological or biochemical makeup of that species (Boerlin and Reid-Smith 2008; Harbottle *et al.* 2006; Tenover 2006). In such cases, all strains of that bacterial species are likewise resistant, or can be potentially resistant, to all the members of those antibacterial classes (Tenover 2006). AmpC β-lactamase genes of Gram-negative bacteria and many MDR efflux systems are examples of intrinsic resistance occurring naturally on the host's chromosome (Alekshun and Levy 2007). Moreover, a decreased binding affinity to the PBPs makes Enterococci intrinsically resistant to cephalosporins (Harbottle *et al.* 2006).

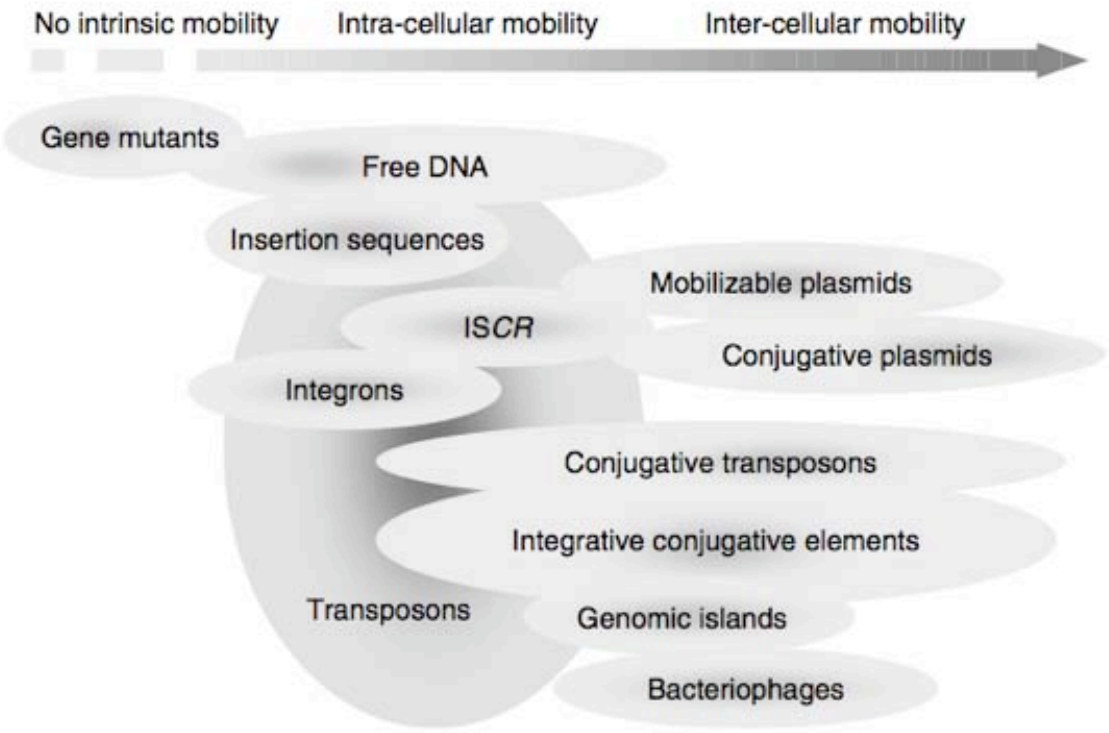


Figure 9 – Different elements involved in the spread of antibiotic resistance genes (Boerlin and Reid-Smith 2008).

Another classic example is the *Mycoplasm* genera, which lacks cell wall thereby is naturally resistant to all the β-lactams, since they have the cell wall as target. This kind of resistance is predictable and should not affect therapeutic options. Although, it does requires laboratory technicians and clinicians possessing a prior knowledge of these mechanisms. Resistant mutants can multiply and emerge out of the overall anonymity due to the interplay between the occurrence of random mutations and selective antimicrobial pressure (Boerlin and Reid-Smith 2008).

1.3.1 Acquired resistance

Extrachromosomal elements are the predominant factor for the escalation of antibiotic resistance. The capture of genetic information encoding resistance new to the cell from an outside source, usually another bacteria, expands the

genome (Alekshun and Levy 2007; Bennett 2008; Boerlin and Reid-Smith 2008; Tenover 2006; Toleman *et al.* 2006b). Acquired resistance differs from intrinsic in that the former is not present in the entire species but only within a certain lineage of bacteria derived from a susceptible parent (Bennett 2008; Harbottle *et al.* 2006). Also, acquired resistance can be divided in vertical and horizontal evolution according to the mechanism of acquisition. The horizontal evolution may occur between strains of the same species or between different bacterial species or genera (Tenover 2006). Acquired mechanisms involve transfer of resistance determinants borne on plasmids, bacteriophages, transposons and also other mobile genetic material. Some bacteria can display both mutations and transfer of resistance, i.e, examples of intrinsic and acquired resistance. This event may occur through one of several processes including transduction (via bacteriophages), conjugation (via plasmids and conjugative transposons) and transformation (via incorporation into the chromosome of chromosomal DNA, plasmids and other DNAs from dead organisms) (Alekshun and Levy 2007; Boerlin and Reid-Smith 2008; Summers 2006).

1.4 Mechanisms of resistance gene transfer

1.4.1 Transformation

Transformation is the longest known mechanism of DNA transfer among prokaryotes (Figure 10). It starts with the release of DNA from a bacterium (donor cell) after death and lyses, or in some bacteria, at a specific point in the growth cycle (Harbottle *et al.* 2006; Summers 2006). Competent recipients can take up the DNA that was release in the surrounding environment after being degraded and broken into smaller fragments. The degraded DNA may include antibiotic resistance genes which can be taken up by nearby bacterium, undergo homologous recombination and incorporated into the bacterial genome (Harbottle *et al.* 2006).

Therefore, heterologous genes would not be able to recombine and would be degraded by the recipient's nucleases, which confines transformation to the transfer of variants (alleles) of shared chromosomal genes among strains of a bacterial species (Summers 2006).

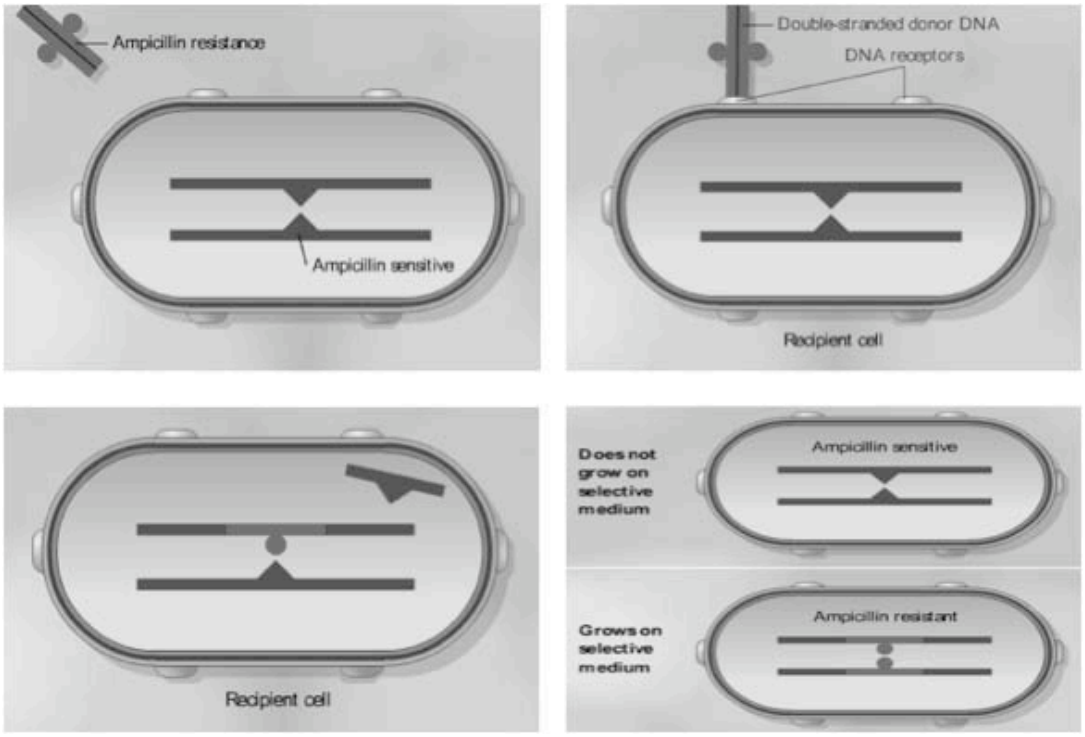


Figure 10 – Description of the alteration of a bacterial cell genome by the uptake of naked, foreign DNA from the surrounding environment. Example of acquisition of ampicillin resistance (Adapted from http://www.1lecture.com/Microbiology/Bacterial%20Transformation/index.html).

1.4.2 Transduction

Transduction is a process carried out by bacteriophages that involves infection of a bacterium, phage replication and packaging of some of the bacterial DNA with the phage DNA (which may include resistance determinants), lysis of that bacterium and infection of subsequent bacteria. Upon subsequent infection, those resistance determinants may be transferred to the infected bacterium (Harbottle *et al.* 2006; Summers 2006).

More specifically, there are two possibilities that bacteriophages can undertake upon infecting a cell: either to integrate into the host's chromosome or to replicate themselves and lyse the cell. The former option sets the phage up to carry some of the host's DNA when it eventually replicates. However, in some cases, the phage accidentally package only host cell DNA when they replicate. In either case, the resulting phage particles attach and inject the DNA they have packaged into a new host. The chromosomal DNA introduced by the phage will be degraded unless it recombines with homologous regions on the resident chromosome (Figure 11). Thus, like transformation, bacteriophage-mediated transduction is usually successful only in transferring alleles of homologous genes among closely related bacteria. And while bacteriophages frequently carry toxins and other virulence factors, it is rare for them to carry antibiotic resistance genes (Summers 2006).

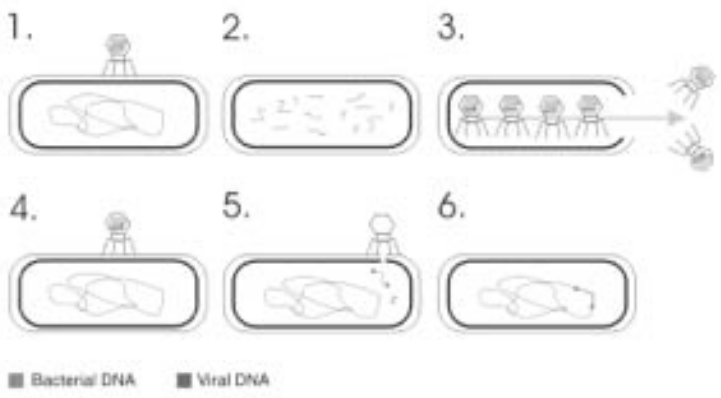


Figure 11 – Transduction process. 1) Phage injects its DNA 2) Phage enzymes degrade host DNA 3) Cell synthesizes new phage that incorporate phages DNA and mistakenly some host DNA 5) Transducing phage injects donor DNA 6) Donor DNA is incorporated into recipient's chromosome by recombination (www.search.com/reference/Transduction_(genetics).

1.4.3 Conjugation

Conjugation is carried out by double stranded DNA plasmids encoding a mating apparatus which includes both combined grappling hook and mating tube called a pilus, as well as a membrane mounted, energy-consuming DNA pump. This mating apparatus transfers a single strand of the plasmid DNA from the donor cell to the recipient cell, where the complementary is synthesized (Figure 12) (Summers 2006). When a resistance plasmid encodes the functions necessary to promote cell-to-cell DNA transfer, particularly their own transfer it is called conjugative. In contrast, others are mobilizable when helped by a conjugative plasmid co-resident in the cell. In general, mobilizable plasmids lack the genes that encode the functions that enable cells to couple prior to DNA transfer but do encode functions needed specifically for transfer. Particularly between Gram-negative bacteria conjugative plasmids tend to be somewhat larger, 30 kb or more, reflecting the sizable amount of DNA (20–30 kb) needed to encode the conjugation functions that permit cell-to-cell coupling (Bennett 2008). An external hair-like transfer appendage, a sex pilus, mediates the coupling, acting like a grappling hook to join donor and recipient cells. To effect envelope-to-envelope contact, when a DNA transfer pore forms to bridge the cytoplasmic compartments of the conjoined cells, the pilus is then retracted into the donor (Bennett 2008)

Conjugative plasmids can either transfer smaller nonconjugative plasmids or integrate into the donor's chromosome and transfer a copy of the entire several megabase molecule to the recipient cell. If the transferred DNA is homologous with the recipient chromosome it can recombine or it can remain associated with the plasmid, which can replicate independently in the recipient as it did in the donor. If the transfer DNA remains associated with the plasmid, this allows for the transfer of genes. These genes, that are completely unrelated to the recipient, still persist in it as they are physically linked with the self-replicating plasmid. Often, plasmids are vehicles of virulence factors including genes facilitating colonization of an animal or plant host and antibiotic resistance genes, notably in the form of transposons and integrons (Summers 2006).

Figure 12 – The conjugation process. Image represents the direct transfer of genetic material between bacterial cells joined by sex pili. (http://evolution.berkeley.edu/evolibrary/)

1.5 Mobile genetic elements

The rapid dissemination of resistance genes among different bacteria, is facilitated by mobile DNA elements which can transport them in tandem (Harbottle *et al.* 2006). Examples, are genes encoding plasmid-mediated β-lactamases that are usually located within or near mobile elements (Arduino *et al.* 2002; Wang *et al.* 2003). While located on bacterial plasmids, at least three fundamentally different recombination systems (transposons, gene cassettes and IS*CR*s), act to assemble and re-assort resistance genes. A particular resistance plasmid may result from any combination, but the operation of all these three mechanisms provides bacteria with an extremely powerful and flexible genetic tool box that is more versatile than previously thought (Bennett 2008; Toleman *et al.* 2006b). The various recombination systems enable bacteria with the necessary mechanisms to capture and re-assort a whole variety of resistance genes that they may need now, and in the future. The movement is random, and generally independent of drug use, but drug use provides a powerful selection for these events once they happen (Bennett 2008).

There are two general types of mobile genetic elements: those that can move from one bacterial cell to another, which in terms of antibiotic resistance includes resistance plasmids and conjugative resistance transposons; and elements that can move from one genetic location to another whitin the same cell. The latter

include resistance transposons, gene cassettes and *ISCR*-promoted gene mobilization (Bennett 2008). The rapid dissemination of antimicrobial resistance among several bacterial genera of human and veterinary importance was facilitated by the spread of mobile genetic elements, in particular plasmids, transposons and integrons (Harbottle *et al.* 2006). Plasmid, transposon and integron-borne resistance to antibiotics now occurs in the majority of Gram-negative pathogens isolated from hospitals and animal production facilities (Gillings *et al.* 2008). Studying the origin of the resistance determinants and the mobile elements that spreads them is critical to the understanding of resistance dissemination and attempting to control future recruitment and spread of resistance genes (Gillings *et al.* 2008).

1.5.1 Plasmids

As mentioned before, horizontal gene transfer involves movement of bacterial genes from one bacterial cell to another. The elements responsible for this movement are bacterial plasmids, specifically conjugative plasmids, which are able to promote their own transfer and the transfer of other plasmids from one bacterial cell to another (Bennett 2008; Carattoli *et al.* 2005). Plasmids are described as extra-chromosomal circular fragments of DNA (Figure 13) that exist separately from the main bacterial chromosome, and are capable of chromosome independent replication.

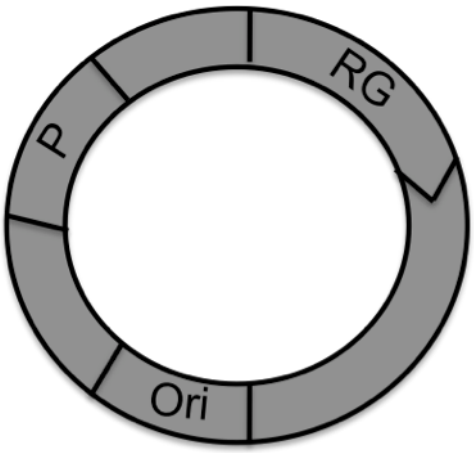


Figure 13 – Diagram of a plasmid. P - promotor; RG - resistance gene; Ori - origin of replication.

Nevertheless, the host cell provides the majority of replication functions (Bennett 2008; Carattoli *et al.* 2005). Moreover, plasmids are double-stranded DNA molecules, present in nearly all bacterial species, that vary in size from a few (7-8 kb) to more than several hundred (>400 kb) kilobase pairs (Carattoli *et al.* 2005).

As it has been observed, particular environmental circumstances may trigger the bacteria to use resources often provided by plasmids which carry useful genes (Bennett 2008; Harbottle *et al.* 2006). Hence, even though they are not essential for survival, plasmids can carry a considerable variety of genes, such as those conferring antibiotic resistance and resistance to a number of toxic heavy metals. Plasmids can also carry genes either providing enzymes that expand the nutritional ability of the cell or virulence determinants that permit invasion of and survival in animal systems, as well as functions that enhance the capacity to repair DNA damage (Bennett 2008; Carattoli *et al.* 2005). Plasmids carrying resistance genes are referred as R plasmids or R factors. Since their discovery in the 1950s, antimicrobial resistance plasmids have been increasingly found associated with both Gram-positive and Gram-negative bacterial pathogens and commensal organisms (Harbottle *et al.* 2006).

The window of therapeutic options became narrow as plasmid-encoded antibiotic resistance encompasses most, if not all classes of antibiotics currently in clinical use, and includes resistance to many that are at the forefront of antibiotic therapy. A single plasmid can simultaneously mediate resistance to multiple first choice antibiotics such as cephalosporins, fluoroquinolones and aminoglycosides, and be shared among different bacterial genera (Bennett 2008; Harbottle *et al.* 2006).

Since plasmids are very promiscuous elements, understanding the molecular epidemiology of resistance plasmids has proven to be a complex task (Hopkins *et al.* 2006). A formal scheme of plasmid classification is based on incompatibility (Inc) groups (Harbottle *et al.* 2006; Walsh 2006). Based on this scheme, their relatedness can be demonstrated either by restriction fragment pattern analysis or by classification into incompatibility groups (Inc) and replicon (rep) typing (Hopkins

et al. 2006). The origin of replication can be considered an additional marker for the constant backbone of the plasmid. Therefore, the association of replicons with specific plasmid borne resistance genes opens the possibility of easily detect and trace the diffusion of successful plasmids as well as the mobilization capability of a resistance gene among different plasmids (Hopkins *et al.* 2006).

The introduction, by conjugation or transformation, of a plasmid of an unknown Inc group into a strain carrying a plasmid of a known Inc group is the basis of incompatibility grouping (Carattoli *et al.* 2005). Plasmids with the same replication control are termed “incompatible”, whereas plasmids with different replication controls are “compatible”. On this basis, two plasmids belonging to the same Inc group cannot be propagated within the same cell line (Carattoli *et al.* 2005).

1.5.2 Transposons

Transposons are among the structures that can be carried by plasmids and carry themselves resistance genes. They can be described as jumping gene systems moving both intra- and inter-molecularly, as they can move from one location on the chromosome to another or from the chromosome to a transmissible plasmid (Alekshun and Levy 2007; Bennett 2008; Harbottle *et al.* 2006).

Transposons appear in many forms, distinguished by structure, genetic relatedness and mechanism of transposition, and can carry a multiplicity of resistance genes (Bennett 2008). Small cryptic elements called insertion sequences (IS), transposons and transposing bacteriophages, such as bacteriophage Mu, fit into a set of mobile elements called transposable elements (Bennett 2008).

Despite belonging to the same set, these elements display some differences between them. For instance, a transposon differs from an IS in size and also because the former encodes at least one function that changes the phenotype of

the cell in a predictable fashion, for instance a resistance transposon confers resistance to a particular antibiotic(s) (Bennett 2008). Transposons are composed by an insertion sequences (IS), intervening DNA and the enzyme responsible for the transposition, called transposase (Figure 14). The terminal regions that participate in recombination specify a protein (e.g., transposase or recombinase) that allows the incorporation into and from specific genomic regions.

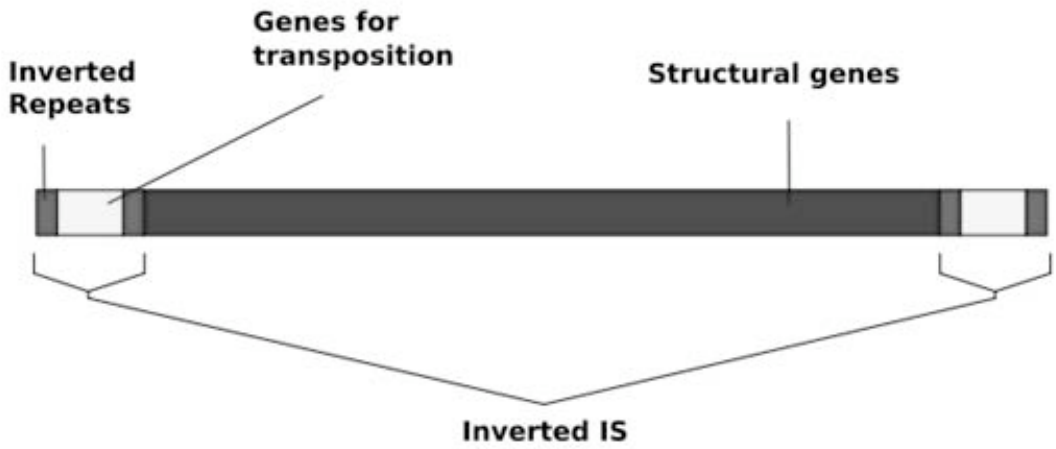


Figure 14 – Schematic structure of transposon composed by inverted repeats, genes for transposition and structural genes (plantstudents.blogfa.com/post-130.aspx).

This jumping set of genes encodes the enzymes and carries the genetic sequences required for movement located within the transposon to randomly jump from one genetic location to another (Harbottle *et al.* 2006). These structures that present this composition and can transmit a large number of resistance determinants in many different bacterial species, are called composit transposons.

For each transposon there is: **a pair of IS elements** flanking the central region are very similar in sequence and can be either as direct repeats, offering the opportunity to migrate to another site, or inverted repeats, more genetically stable, to the section containing genes conferring antibiotic resistance; **a central DNA sequence** the expression of which alters the cell phenotype, yet it is not inherently

able to transpose or mediates transposition; and a **transposase**, that promotes transposition (Bennett 2008; Harbottle *et al.* 2006).

Transposition to another location occurs by a two-stage homologous recombination process. At first, a circular, double-stranded DNA species comprising the central section of the composite resistance transposon and one copy of the IS element are released. This process occurs by the excision of part of the composite structure from its existing site by single crossover between the copies of the IS element, whereas one is released and the other remains at the original genetic location (Bennett 2008). A composite transposon can be recreated in a new location when the released DNA is rescued, essentially by reversing the first recombination, by homologous recombination involving a single crossover, using the copy of the IS sequence on the free intermediate and another copy at the new location (Bennett 2008). Moreover, the movement to the new location can be accomplished by insertion into a conjugative plasmid or via a conjugative transposon, being the latter unique as they have behave as plasmids and can facilitate the transfer of endogenous plasmids from one organism to another (Alekshun and Levy 2007; Harbottle *et al.* 2006). Unlike plasmids, the covalently closed single-stranded DNA circular structure that conjugative transposons form after promoting their excision from the donor cell's genome, does not replicate except to synthesize a complementary strand to become double stranded. These transposons then promote their conjugation to a neighbouring bacterium and following conjugation, integrate into the recipient chromosome or the recipient host's plasmid (Harbottle *et al.* 2006).

Tn*5* and Tn*10* are well-known example of resistance transposons, encoding resistance to aminoglycosides such as kanamycin and neomycin, and resistance to tetracycline, respectively, and both can be found in a number of Gram-negative bacteria (Bennett 2008).

Such structures are created by chance but conferring a distinct survival advantage, when they become established in the population. For instance, the selective forces operating on the bacterial flora, such as the exposure to

kanamycin/neomycin or tetracycline respectively, may prompt for such establishment (Bennett 2008). Tn*3* and Tn*21*, are examples of complex transposons commonly found in plasmids among Enterobacteriaceae. Tn*3*, encoding resistance to a number of β-lactams antibiotics, including ampicillin, and Tn*21*, encoding resistance to streptomycin, spectomycin and sulphonamides as well as mercuric ions (Bennett 2008). Some aspects of Tn*21* construction can be deduced; however, the construction of this type of element is less easily explained and no general evidence-based model has been proposed yet.

Complex transposons are probably the result of multiple recombination events. These events include both insertions and deletions, which first insert non-transposition functions into a cryptic element and then refine the sequence by deletion to eliminate 'non-essential' functions (Bennett 2008). It has been verified that increasing the size of a particular transposable element reduces its frequency of transposition, therefore this refinement would assemble a compacted element and hence more readily transposable (Bennett 2008).

1.5.3 Integrons

Historically, transposons were considered to be the main vehicles of gene transfer between DNA molecules. Nevertheless, structures of some resistance transposons, such as Tn*21*-like transposons, are not fixed. For instance, it was discovered that they contain gene integration systems named integrons, capable of capture and re-assort individual resistance genes (Collis and Hall 1995). Hence, this novel system of antibiotic resistance genes movement, explains the magnitude of resistance development and dissemination (Harbottle *et al.* 2006). Integrons are mobile gene capture systems that utilize site-specific recombination instead of transposition mechanisms to integrate at a specific location (Bennett 2008; Harbottle *et al.* 2006). A recombination system consisting of a gene, *int*, which encodes a site-specific recombination enzyme referred as integrase, and a proximal primary recombination sequence refered as *attI* site, compose the

functional platform of an integron. The integrase mediates recombination between the *attI* site and a secondary target named an *attC* site (or 59-base element [59be]) (Bennett 2008; Rowe-Magnus and Mazel 2001; Toleman *et al.* 2006b; Walsh 2006). The *attC* site is generally associated with a single Open Reading Frame (ORF) located in a structure termed gene cassette (Rowe-Magnus and Mazel 2001). A general representation of class 1 integrons is represented in Figure 15. The variety of gene cassettes present in the natural environments is massive, amounting thousands of distinctive cassettes (Gillings *et al.* 2008). Those exist as small, free circular DNA structures around 500-1000 bp, that are not expressed due to the lack of the promoter region (Toleman *et al.* 2006b).

This condition is a transitional state within the mechanism that mediates cassette transfer from one integron to another or the reposition of gene cassettes within a particular integron (Bennett 2008; Harbottle *et al.* 2006). Downstream of the promoter-less resistance gene is often a 59 bp element that acts as the recombination site. The expression of a gene encoded by a cassette is facilitated by the insertion of the latter into the integron structure via the recombination process at the *attI* recombination site downstream from a promoter (Bennett 2008; Harbottle *et al.* 2006; Rodriguez-Martinez *et al.* 2006). Gene cassettes may generate remarkable resistance arrays being inserted one after the other into the integration site and after each insertion *attI* regenerates (Bennett 2008). Two or more are expressed in a polycistronic manner from the cassette promoter within 5'-CS and such expression displays polarity. The order of the gene cassettes indicates the order of their addition being the one near the 5'-CS the latest addition, because each cassette is inserted at the same point (Bennett 2008).

Integrons can be distinguished according to their integrase sequence and several classes of integrons have been reported. Class 1 integrons are the most frequently identified, specially among enteric isolates and also in a number of different genera (Harbottle *et al.* 2006). Class 1 integrons, have distinctive structure comprising two terminal invariable regions, termed conserved sequences (CS), and a highly variable central section (Collis and Hall 1995). As it has been referred while describing integrons in general, at one end is the 5'-CS, which

accommodates *int*, the cassette insertion site *attI* and the promoter from which cassette genes are expressed. At the other end is the 3'-CS, composed by part of the *qacE*Δ1 gene, that when intact confers resistance to quaternary ammonium compounds, followed by *sul*, a gene conferring resistance to sulphonamides (Partridge and Hall 2003). The gene cassettes of a particular integron are flanked by these CS regions originating the so-called variable region, being variable both in terms of length and sequence. This region necessarily varies as the identities and number of the gene cassettes changes from one integron to another (Collis and Hall 1995) .

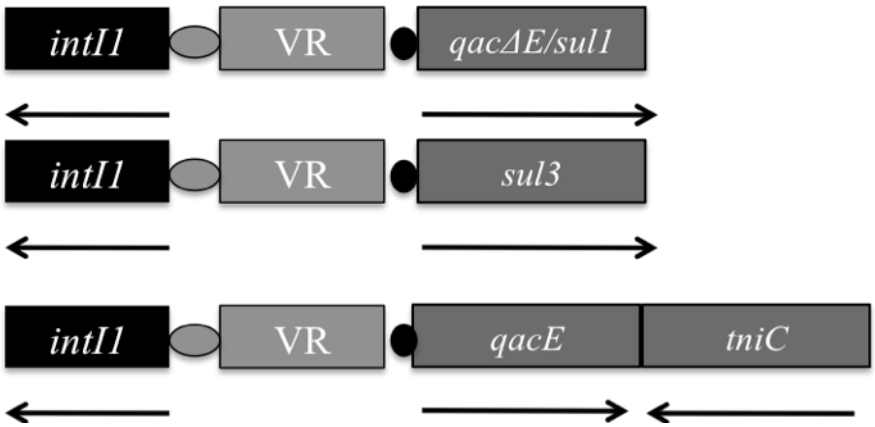


Figure 15 – Structure of a class 1 integrons. Open reading frames are represented by boxes, with the arrows indicating the direction of transcription. Solid black circles represent 59-base elements, and silver ellipses represent the *att*I1 site of the integron.

Integrons are not static structures rather they are constantly evolving in order to provide bacteria with the tools that enable them to resist to newer antimicrobials. In addition, recent findings on integron diversity and evolution suggest that they are much more diverse than originally thought (Boerlin and Reid-Smith 2008). Large chromosomal arrays of gene cassettes encoding a diversity of functions, known as super integrons and containing hundreds of gene cassettes, including resistance gene cassettes, have been found in a number of bacterial species. However, where individual cassettes derive from is often unknown (Bennett 2008). The term "super integron" was introduced by Mazel *et al.* in 1998 (Mazel *et al.* 1998), to describe integrons that have incorporated hundreds of gene cassettes. A

classical example is the superintegron (SI) that has been found in the *Vibrio cholerae* chromosome, which is 179kb long, and harbours 179 gene cassettes. Superintegrons are likely to play an important role in bacterial evolution. However the role played by the superintegron has not been fully investigated (Harbottle *et al.* 2006). A potential selection of superintegrons among other bacterial pathogens can be driven from their complex structure, presence of multiple gene cassettes, chromosomal integration, and possible co-integration of virulence factors (Harbottle *et al.* 2006). Usually, and although class 1 integrons contain less than five gene cassettes, the individual cassettes in the superintegrons arrays can migrate to these smaller integrons found on plasmids present in bacteria of clinical origin, expanding their variability (Bennett 2008; Gillings *et al.* 2008). The ability of class 1 integrons to gather diverse genes and their inherent mobility has led to their important role in the spread of resistance determinants through human ecosystems (Gillings *et al.* 2008)

1.5.4 Insertion Sequence Common Regions (IS*CRs*)

Scaling down the variety of genetic mobile elements based on their size, there are smaller elements than the above described, to which special attention should be given.

Although the movement of resistance transposons and gene cassettes can account for much of the recombination involved in resistance plasmid construction, they fail to explain the spread of a substantial and growing subset of resistance (Bennett 2008; Toleman *et al.* 2006b). Recently, it has become perceptible the contribution of at least another recombination system to the assembly of banks of resistance genes on bacterial plasmids (Bennett 2008; Toleman *et al.* 2006b). These recombination systems involved in this aspect of plasmid evolution are referred as Insertion Sequences *Common Region* or IS*CR* elements (Bennett 2008). When a specific sequence is found in different genetic locations it may be thought to be a transposable element. When that sequence is linked with a multiplicity of resistance genes, suspicion hardens that it is responsible for the movements of those genes (Toleman *et al.* 2006b).

IS*CR* elements were first described as sequences associated with class 1 integrons, yet somewhat distinct from them. The same sequence was commonly found downstream of the 3' CS of class 1 integrons, therefore it became known as the common region, or CR, and termed in order to distinguish it from the 5' and 3'CS of class 1 integrons (Bennett 2008; Toleman *et al.* 2006b). In the early 1990s the first common regions, were discovered and reported. They were described as a DNA sequence of 2,154 bp, accommodating a single ORF and found in two complex class 1 integrons, In*6* and In7 (Toleman *et al.* 2006b). Following a comparative analysis, it was perceptible that these CRs displayed a certain degree of relatedness to each other and bear a resemblance to an atypical class of insertion sequences, designated IS*91*-like (Chen *et al.* 2008; Toleman *et al.* 2006b; Toleman and Walsh 2008; Walsh 2006). It is known for some time that IS*91*-like elements differ distinctly from most other IS elements in both structure and mode of transposition, and recently the IS*CRs* were found to exhibit the same characteristics (Bennett 2008). On the one hand, they lack terminal inverted repeats (IR), but possess distinct terminal sequences designated *ori*S and *ter*S (Figure 16), that indicates the unique sites for the initiation and the termination, respectively, of the mechanism which by they are transposed (Bennett 2008; Harbottle *et al.* 2006; Toleman *et al.* 2006b).

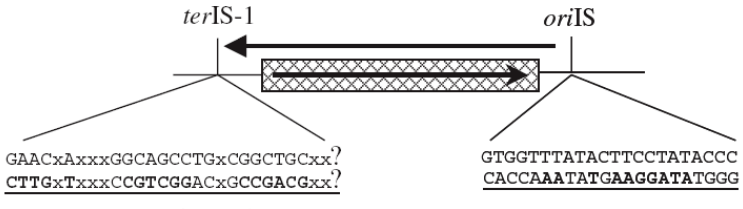


Figure 16 – *ori*S and *ter*S-1 are denoted as the insertion and termination sites of IS*CR1* transposition, respectively. Sequences underlined read 5'->3' for each site and the nucleotides in bold match those of the consensus sequence of IS*91*, IS*1294* and IS*801* The larger bold horizontal arrow indicates the direction (from right to left) and origin of replication (Toleman *et al.* 2006b).

One feature of these systems is that they seem to insert relatively randomly in any DNA molecule, meaning that virtually they can be inserted adjacent to any gene and transpose it. Moreover, and very important in the dissemination of antibiotic resistance genes, is that the termination mechanism of IS*CR*s replication is not very accurate, showing a degree of inaccuracy up to 10%, allowing replication to proceed beyond *ter*IS and into adjacent sequence, where it appears to be terminated more or less at random (Bennett 2008; Harbottle *et al.* 2006). On the other hand, once the movement of these elements involves rolling-circle (RC) replication, therefore allowing rearrangements that may not be possible by traditional rearrangements, it is thought that these elements transpose by a mechanism termed RC transposition, (Harbottle *et al.* 2006). As a consequence, IS*91*-like elements can transpose adjacent DNA sequences, mediated by a single copy of the element, as the precedent given by IS*1294*, which was shown to mobilize the adjacent kanamycin-resistance gene by a single copy (Toleman *et al.* 2006b; Walsh 2006).

The mobilization is intrinsic to the RC transposition mechanism and will, in principle, mobilize any DNA following the replication terminal signal. Integrons that differ from the more common form of class 1 integrons, in the way that contain two copies of the 3´CS and a CR, are called complex class 1 integrons and their construction was predicted to be based on the RC transposition mechanism (Bennett 2008; Toleman *et al.* 2006b). Furthermore, it has also been argued that IS*CR*s are associated with the mobilization of virtually every class of antibiotic resistance gene, including those encoding extended-spectrum β-lactamases (ESBLs), carbapenemases, enzymes conferring broad-spectrum aminoglycoside resistance, as well as genes encoding resistance to floranphenicol/chloramphenicol, trimethoprim and quinolones (Toleman *et al.* 2006b).

Another interesting feature of IS*CRs* is that they are intimately associated with different resistance genes carried on each integron. In the case of In*6*, the CR is found immediately upstream of a chloramphenicol resistance gene (*catAII*), while the CR in In*7* is associated with a novel trimethoprim resistance gene (*dfrA10*)

(Toleman *et al.* 2006b). A typical circumstance for the overwhelming majority of resistance genes embedded in class 1 integrons is their linkage with the 59 base elements (site-specific recombination sequences that define gene cassettes). However it was noticed with striking surprise that neither of these two CR-associated resistance genes was linked to a 59 base elements as it would be expected (Toleman *et al.* 2006b). Therefore, a model was proposed, in which IS*CR1* became associated with the 3'-CS of a class 1 integron, as a chance event which was followed by a second but aberrant transposition event that placed IS*CR1*, together with different lengths of the class 1 integron, alongside a variety of resistance genes (Bennett 2008; Toleman *et al.* 2006b).

The RC transposition mechanisms generated free circular species containing IS*CR1*, whole or part of 3'-CS and a non-gene resistance cassette gene(s), from the former constructs. These intermediates were subsequently rescued by homologous recombination into other class 1 integron, using the common 3'-CS sequence as point of crossover (Bennett 2008). The process is almost complete and will be finalized with the mobilization of these constructs onto a conjugative plasmid and then via conjugation, into other species/genera of bacteria as shown in Figure 17 (Bennett 2008; Toleman *et al.* 2006b). The striking potential for movement between bacterial species displayed by IS*CR* elements became perceptible based on the observation that several, if not all, IS*CR* elements have migrated on to plasmids. Accordingly, a large percentage of the entire bacterial gene pool on the planet is potentially available for transposition on to bacterial plasmids, mediated by the IS*CR* (Bennett 2008). Since the first CR sequence was identified, several more have been discovered worldwide and IS*CR*s have now been found to be present on both plasmids and chromosomes in numerous Gram-negative organisms of clinical importance, and in a few Gram-positive bacteria (Toleman *et al.* 2006b).

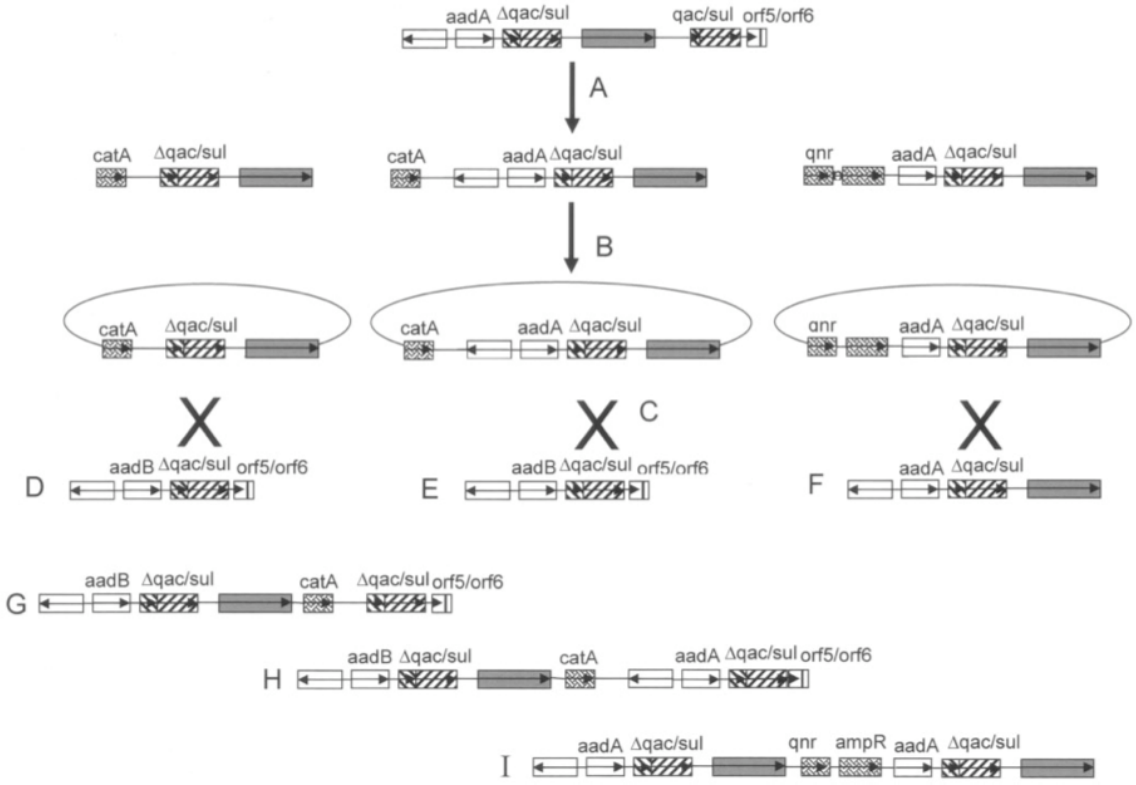


Figure 17 – Model of IS*CR1*-mediated construction of complex class 1 integrons. The construction of complex class 1 integrons can be explained by a three step mechanism. (A) Aberrant RC replication of the IS*CR1* element (fused to 3'CS) generates transposition intermediates of different length. These intermediates then transpose adjacent to an antibiotic resistance gene (e.g. *catA* or *qnr*), in another location. (B) A second aberrant RC replication event produces circular intermediates which now include *catA* or *qnr*. (C) These circular intermediates can then be rescued by recombination events between 3'CS on another 'normal' class 1 integron (D and E) producing the complex integrons G and H, or they can be rescued by (F) a class 1 integron already including a copy of IS*CR1* generating the complex integron I. Such aberrant RC transposition and recombination rescue events provide an explanation for the spectrum of complex class 1 integrons observed in nature. Boxes represent the open reading frames of the various genes with arrows indicating the direction of their transcription. The open reading frame of the IS*CR1* elements is shaded in grey and the resistance genes that lack a 59 base element are patterned.

As already mentioned, IS*CRs* are notable for their close association with a wide variety of antibiotic resistance genes. At present, there are at least 19 members of the IS*CR* family, and all adjacent to genes that are not the natural complement of the host cell, the vast majority being antibiotic resistance genes (Toleman and Walsh 2008). Generally, they can be divided into two groups: those that form complex class 1 integrons (termed IS*CR1*), and those that are associated with non-class 1 integrons (IS*CR2-19*) (Toleman *et al.* 2006b).

IS*CR1* it has been reported in association with genes encoding either resistance to chloramphenicol (*cat*AII), trimethoprim (*dfr*A10, *dfr*A23, *dfr*A3b, *dfr*A19) or aminoglycosides (*arm*A), as well as with class A β-lactamases ($bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}9}$, $bla_{CTX\text{-}M\text{-}20}$, $bla_{PER\text{-}3}$, $bla_{VEB\text{-}3}$) and class C β-lactamases ($bla_{DHA\text{-}1}$, $bla_{CMY\text{-}1}$, $bla_{CMY\text{-}8}$, $bla_{CMY\text{-}9}$, $bla_{CMY\text{-}10}$, $bla_{MOX\text{-}1}$) (Arduino *et al.* 2002; Boyd *et al.* 2002; Di Conza *et al.* 2002; Partridge and Hall 2003; Sabate *et al.* 2002; Su *et al.* 2008; Verdet *et al.* 2000). The recently discovered gene, *qnr,* is also closely linked to IS*CR1* specially the *qnrA1* allele (Chen *et al.* 2008; Garnier *et al.* 2006; Nordmann and Poirel 2005).

IS*CR2* (encompassing the gene known as *orfA*) is the primary representation of the second subgroup of IS*CR* elements. It is part of the MDR-encoding genetic element SXT, and is also encountered on numerous plasmids carrying chloramphenicol/florfenicol and sulfonamide resistance (Toleman *et al.* 2006b). While IS*CR1* has until now been found as a component of complex class 1 integrons, IS*CR2* has never been associated with class 1 integrons but often associated with the *sul2* gene (Toleman *et al.* 2006b). Nevertheless, its linkage with genes encoding resistance to trimethoprim (*dfr*A18, *dfr*A9, *dfr*A20) and tetracycline (*tet*R) has been reported.

All the others IS*CRs* were described in particular genetic contexts, scattered worldwide and in different species. For instance, IS*CR3* typifies the third subgroup of IS*CR* elements that as it has been happened before to other elements, was originally identified by the transposase gene, namely, *orf2*. To date, it has been associated with the SGI1 element (and variants thereof) and is also linked to an *erm* gene encoding erythromycin resistance and the *rmtB* gene enconding aminoglycoside resistance (Toleman *et al.* 2006b). *qac, dfr*A10, *ere*B, *yie*E and *yie*F are other examples of genes associated with IS*CR3* . IS*CR4,* was first identified by its transposase gene, previously named *orf495* (Poirel *et al.* 2004). IS*CR4* was associated with the MBL gene $bla_{SPM\text{-}1}$, found on a large plasmid in various strains of *P. aeruginosa*, capable of hydrolyzing all β-lactam antibiotics except from aztreonam (Murphy *et al.* 2003; Toleman *et al.* 2002; Toleman *et al.* 2006b). IS*CR5* is to date uniquely associated with the class D oxacillinase gene

bla$_{OXA-45}$, found in a *P. aeruginosa* strain 07-406, which was collected via the SENTRY Antimicrobial Surveillance Program from the Anderson Medical Center in Texas (Toleman *et al.* 2006b). IS*CR6* was found in six epidemiologically unrelated clinical strains of *P. aeruginosa* that were isolated between 1992 and 1998 in Bulgaria. In these strains, IS*CR6* is adjacent to an aminoglycoside 4'-O-adenylyltransferase encoded by the *ant(4')-IIb* gene, conferring resistance to amikacin but not to netilmicin (Sabtcheva *et al.* 2003; Toleman *et al.* 2006b).
IS*CR7* and IS*CR8* were identified by sequences that appear to be incomplete. Both were found in *Pseudomonas spp*., close to genes encoding degradative enzymes involved with catabolism of haloalkanes and monocyclic nitroaromatic compounds respectively. Both putative transposase genes appear to have suffered a deletion and sustained double insertions, in comparison to other IS*CR* genes, suggesting that both transposases are inactive (Toleman *et al.* 2006b). IS*CR9* and IS*CR10* were discovered in strains of *S. maltophilia* (Toleman *et al.* 2006b). IS*CR11* was discovered in two *A. baumannii* isolates from Germany that have the MBL *bla*$_{VIM-2}$ and in a *P. aeruginosa* isolate from Greece that produces MBL *bla*$_{VIM-1}$ (Toleman *et al.* 2006b). IS*CR12* was found in a *P. aeruginosa* strain that produces the MBL SPM-1 and carries two IS*CR* elements, IS*CR4* and IS*CR12* (Toleman *et al.* 2006b). IS*CR13* was identified in the *A. hydrophila* genome and displays approximately 54% nucleotide identity to IS*CR5*. IS*CR13* was found adjacent to a gene with high identity to an aminoglycoside resistance gene (Toleman *et al.* 2006b).

1.6 Scope

Antibiotic resistance has become dramatic worldwide, specially in the vast majority of the hospitals. Until recently, within the hospital context, species posing problems for the treatment of patients were relatively well known. However, nowadays any species can be a potential problem if under the favorable selective pressures and/or when infecting immunocompromized patients.

Moreover, the antibiotic resistance has breached outside of the hospital environment due to an inappropriate use of antibiotics. The consequences of this misuse, mostly in the veterinary, is not yet very clear but apparently, as it happened in the hospital environment, it seems that soon it will be catastrophic.
The mechanisms involved in the antibiotic resistance are many and in constant adaptation to the pressures exerted from the surrounding environment. A special attention must be given to the factors potentiating horizontal gene transfer (HGT) as the consequences of the transferred resistance are less predictable.

An accurate and detailed study of the prevalent resistance determinants, either being resistance genes or structures enhancing/involved in their dissemination is needed in order to provide a more realistic scenario. This knowledge will be helpful to the clinicians and also to the infection control commissions to implement new measures. Therefore all the information gathered in research laboratories is of the utmost importance and should be taken into consideration by the clinicians whenever it is possible.

1.6.1 Main goal of this thesis

The main goal of this thesis is to contribute to the knowledge of IS*CR*s distribution and prevalence in selected species collected within the hospital environment. Microoganisms were isolated from samples, collected at Hospital Infante D. Pedro, EPE in Aveiro. The study focuses on the association of IS*CRs* with antibiotic resistance genes, in resistant and MDR isolates. Since, natural environments are also important reservoirs of antibiotic resistance genes (Baquero *et al.* 2008b) environmental microorganisms (Gram-negative isolates) were also collected (from water and fish guts samples) and screened for the presence of the IS*CR* elements.

The thesis is divided in five chapters as follows:

In chapter 1, 210 non-biased *Escherichia coli* recovered from three separate patient-cohorts in the Hospital Infante D. Pedro, EPE: day care (DC), emergency room (ER), and long term inpatients (IP) were studied. These isolates were collected from various clinical specimens and their resistance profile was determined. The occurrence of IS*CR1* elements, integrons and β-lactamase genes was also studied.

In chapter 2, a *Klebsiella pneumoniae* population collected in 2007 from various clinical specimens of inpatients of the Hospital Infante D. Pedro EPE was studied. The resistance profile of these isolates was determined and the occurrence of IS*CR*s and resistance genes associated with them was investigated.

Chapter 3, describes a MDR *Citrobacter freundii* collected in 2008 and recovered from an inpatient in the Hospital Infante D. Pedro EPE. The resistance profile of this isolate was determined and the occurrence IS*CR1* elements, integrons and β-lactamase genes was investigated.

In chapter 4, MDR *Acinetobacter baumannii* isolates were screened for the presence of IS*CR* elements and their genetic context studied. The microorganisms were recovered during an outbreak, in 2006, from various biological products of inpatients from the Hospital Infante D. Pedro. For comparative purposes, an Argentinean population, known to possess the IS*CR2* element, was included.

Chapter 5 aimed to show from an environmental perspective the occurrence and dissemination of IS*CR*s in environmental bacteria. For that purpose, microorganisms were collected from geographical distant locations (Ria de Aveiro, Portugal and Cardiff lakes, UK) and also from different sources (Water and fish guts).

2. Material and Methods

2.1 Bacterial strains

The clinical isolates used in this study were collected, in different timeframes, from various clinical specimens (urine, pus, sputum, blood and others) from patients of the Hospital Infante D. Pedro EPE, Aveiro, Portugal. The microorganisms studied include: *E. coli, K. pneumoniae, A. baumannii* and *C. freundii*.

For comparative studies, Libyan and Argentinean clinical isolates available at Prof. Timothy R. Walsh laboratory, Dept. Medical Microbiology, Cardiff University, Heath Park, UK, were included.

For the screening of mobile genetic elements in environmental microorganisms, isolates were collected from:
a) Fish guts (*Liza aurata)*: the animals were captured from three different sampling sites with different levels and sources of contamination. The sites were S. Jacinto, (a clean reference site), Largo do Laranjo (heavy metals contamination) and Vagos (agriculture and aquacultures). The guts were recovered from fifteen animals and placed in a 50 mL tube with 25 mL of Tryptic Soy broth (TSB, Merck, Germany) overnight at 37 ºC. 100 μL of the samples were spread in Gram-negative selective medium plates, Mackonkey agar (Mck, Merck, Germany) and incubated overnight at 37 ºC. Colonies with distinct morphology were selected, purified on plates containing the same medium and stored at -80 ºC in microtubes with 400 μL of TSB and 200 μL glycerol at 40%.
b) Water: isolates were kindly provided by Prof. António Correia, Laboratory of Microbiology (Microlab), University of Aveiro, Portugal.

Positive controls used in the present study were kindly supplied by Beatrice Berçot, Centre Hospitalier de Bicêtre, Service de Bactériologie-Virologie-Parasitologie-Hygiène, Le Kremlin-Bicêtre, France and Prof. Gabriela Jorge da Silva, Faculdade de Farmácia da Universidade de Coimbra, Coimbra, Portugal.

2.2 Antibiotics stock solutions

1. **Chloramphenicol**: 30 mg/mL dissolved in 70% ethanol and stored at -20 ºC.

2. **Nalidixic acid:** 30 mg/mL in 300 mM NaOH and stored in aliquots at -20 ºC. **Ciprofloxacin:** 2 mg/mL infusion (Claris Lifesciences, UK)

3. **Sulphamethoxazol:** 30 mg/mL in 0.1 M NaOH, filter sterilised and stored in aliquots at -20 ºC.

4. **Ampicillin:** 100 mg/mL dissolved in distilled water, filter sterilised and stored in aliquots at -20 ºC.

4. **Sodium azide**: 200 mg/mL dissolved in distilled water and stored at -20 °C.

2.3 Polymerase Chain Reaction (PCR) amplifications

PCR reactions were performed using heat-denatured cells from pure cultures or DNA as substrate. 20 μL reaction mixtures contained at a final concentration: 1X PCR buffer, 3 mM $MgCl_2$, 5% dimethylsulfoxide, 200 μM each nucleotide, 0.3 pmol/μL of each primer, 1 U of *Taq* polymerase, and 1 μL of heat-denatured cells or 50 -100 ng of purified DNA.
Different PCR annealing temperatures (Table 2) were used according the gene to amplify, but based on the general program described below and during 30 cycles:

Initial Denaturation	95 ºC for 10 min
Denaturation	95 ºC for 1 min
Annealing	Variable 1 min
Elongation	68 ºC for 2 min
Extension	68 ºC for 5 min

Table 2 – Gene, primers sequence, annealing temperature and size of the expected fragments

Gene	Primers sequence (5'-3')	Annealing	Size (bp)	Reference
bla$_{TEM}$	**F-**AAAGATGCTGAAGATCA **R-**TTTGGTATGGCTTCATTC	44 ºC	425	(Speldooren *et al.* 1998)
bla$_{SHV}$	**F-**GCGAAAGCCAGCTGTCGGGC **R-**GATTGGCGGCGCTGTTATCGC	62 ºC	304	(Henriques et al. 2006)
bla$_{CTX-M-15}$	**F-**AGAATAAGGAATCCCATGGTT **R-**ACCGTCGGTGACGATTTTAG	55 ºC	538	(Mendonça *et al.* 2006)
bla$_{IMP}$	**F-**GAATAGAGTGGATTAATTCTC **R-**GGTTTAAYAAAACAACCACC	55 ºC	232	(Henriques *et al.* 2006)
bla$_{VIM}$	**F-**GATGGTGTTTGGTCGCATATCG **R-**GCCACGTTCCCCGCAGACG	58 ºC	475	(Henriques *et al.* 2006)
bla$_{OXA}$ [1]	**F-**ACACAATACATATCAACTTCGC **R-**AGTGTGTTTAGAATGGTGATC	53 ºC	814	(Ouellette *et al.* 1987)
bla$_{OXA-23}$	**F-**GGAATTCCATGAATAAATATTTTACTTGC **R-**CGGGATCCCGTTAAATAATATTCAGGTC	55 ºC	840	(Heritier *et al.* 2005)
bla$_{OXA-40}$	**F-**GGAATTCCATGAAAAAATTTATACTTCC **R-**CGGGATCCCGTTAAATGATTCCAAGATTTTCTAGC	55 ºC	846	(Heritier *et al.* 2005)
int1	**F-**CTGGATTTCGATCACGGCACG **R-**ACATGCGTGTAAATCATCGTCG	65 ºC	500	(Barlow *et al.* 2004)
VR[2]	**F-**GAACCTTGACCGAACGCAG **R-**AGCTTAGTAAAGCCCTCGCTAG	59 ºC	Variable	(Barlow *et al.* 2004)
ISEcp1[3]	**F-**AAAAATGATTGAAAGGTGGT	55 ºC	1200	(Eckert *et al.* 2004a)
IS*aba*1	**F-**ATGCAGCGCTTCTTTGCAGG **R-**AATGATTGGTGACAATGAAG	55 ºC	389	(Héritier *et al.* 2006)
*qnr*A	**F-**GGGTATGGATATTATTGATAAAG **R-**CTAATCCGGCAGCACTATTA	55 ºC	660	(Cattoir *et al.* 2007)
*qnr*B	**F-**GATCGTGAAAGCCAGAAAGG **R-**ACGATGCCTGGTAGTTGTCC	60 ºC	469	(Robicsek *et al.* 2006)
*qnr*S	**F-**AGTGATCTCACCTTCACCGC **R-**CAGGCTGCAATTTTGATACC	60 ºC	550	(Cattoir *et al*. 2007)
IS*CR1*	**F-**GGAGTGACGGGCACTGGCTG **R-**CACTCGTTTACCGCTCAAGC	55 ºC	750	This study
IS*CR2*	**F-**CACTGGCTGGCAATGTCTAGC **R-**GAGTCAACTGCGGTCCAAAGC	55 ºC	1800	This study
*flo*R	**F-**TCGACATCCTCGCTTCACTG **R-**ACCTCGTGAATGTCGGTATCC	55 ºC	1200	This study
*sul*2	**F-**TCTTTCAGCGCCGCCAATAC **R-**GACAGTTTCTCCGATGGAGG	55 ºC	800	This study
*glm*M	**F-**GAGTCAACTGCGGTCCAAAGC **R-**GGCTTTGCCACGAATACCGTCT	55 ºC	250	This study
TNIC[4]	**R-**CGATCTCTGCGAAGAACTCG	55 ºC	Variable	(Toleman *et al.* 2007b)
MOX	**F-**GCTGCTCAAGGAGCACAGGAT **R-**CACATTGACATAGGTGTGGTGC	64 ºC	520	(Perez-Perez and Hanson 2002)
FOX	**F-**AACATGGGGTATCAGGGAGATG **R-**CAA AGCGCGTAACCGGATTGG	64 ºC	190	(Perez-Perez and Hanson 2002)
CMY	**F-**TGGCCAGAACTGACAGGCAA **R-**TTTCTCCTGAACGTGGCTGGC	64 ºC	462	(Perez-Perez and Hanson 2002)
ACC	**F-**AACAGCCTCAGCAGCCGGTTA **R-**TTCGCCGCAATCATCCCTAGC	64 ºC	346	(Perez-Perez and Hanson 2002)
DHA	**F-**AACTTTCACAGGTGTGCTGGGT **R-**CCGTACGCATACTGGCTTTGC	64 ºC	405	(Perez-Perez and Hanson 2002)

[1] *bla*$_{OXA-1}$ derivates; [2] VR – Variable region; [3] Primer to be used in combination with *bla*$_{CTX-M-15}$ reverse primer

[4] Primer to be used in combination with forward primer of VR

All PCRs were carried out with *Taq* polymerase, nucleotides and buffers purchased from ABgene (NewEngland Biolabs, UK) in a Bio-Rad cycler Thermal Cycler (Bio-Rad Laboratories, Richmond, CA, USA). PCR products were separated by electrophoresis in a 1% agarose gel and stained with ethidium bromide (0.5 μg/mL). Gel images were acquired using a Molecular Imager FX^{TM} system (Bio-Rad, Richmond, CA, USA)

2.4 Nucleotide sequence determination and sequence analysis

PCR products were purified with the Qiagen Agarose Gel Extraction Kit (Qiagen, Germany) according to the manufactures instructions and sequenced in both strands by STABVida, Portugal. Sequences were edited manually after examination of the corresponding chromatogram files using the Biological Sequence Alignment Editor Bioedit, version 7.0.0. Similarity to sequences deposited in the EMBL Genbank was searched using the BLAST program (Altschul *et al.* 1997). The alignment of the sequences was performed using the ClustalW program.

2.5 Plasmid DNA Extraction

Bacterial cells were grown overnight in liquid medium, tryptic soy broth (TSB, Merck, Germany). Plasmid DNA was extracted with two different protocols: Qiagen protocol and alkaline lysis. Protocols were performed as follows:

2.5.1 Qiagen protocol

1. 5 mL (or 1 mL) of bacterial culture were centrifuged at 16000 x *g.*
2. Cells were resuspended in 250 μL of P1.
3. 250 μL of P2 were added and the microtubes inverted 4-6 times[1].
4. 300 μL of N3 were added and the solution mixed immediately and thoroughly by inverting the microtube 4-6 times[2].
5. The microtubes were centrifuged at 16000 x *g*.
6. The supernatant was applied to the spin columns and centrifuged for 60 s at 16000 x *g*.
7. The columns were washed by adding 500 μL of PB and centrifuged for 60 s. The flow-through was then discarded.
8. The spin columns were washed with 750 μL of PE and centrifuged for 60 s at 16000 x *g*.
9. The flow-through was discarded and the columns centrifuged for 1 min at 16000 x *g.*
10. The DNA was eluted in 50 μL of elution buffer.

[1.] The solution needs to become viscous; [2.] The solution should become cloudy.

2.5.2 Alkaline lysis

1. Bacterial cells were grown overnight in TSB medium (100 mL) at 37 ºC.
2. Cultures were centrifuged at 16000 x *g*.
3. The precipitate was resuspended in 3 mL of solution I[1]. Lysozyme (25 mg/mL) was added to solution I.
4. The precipitate was transferred to a smaller tube and left 15 min at room temperature.
5. 8 mL of solution II[2] were added to each tube. The top was covered with film and the tube slowly inverted several times and left on ice for 10 min.

6. 6 mL of potassium acetate 5 M, pH 4.8 were added. The top was again covered and the tube inverted. The tubes were left 10 min on ice.
7. The solutions were centrifuged at 16000 x *g* for 30 min at 4 ºC.
8. Six volumes (vol) of isopropanol were added to each tube, mixed and left 15 min at room temperature.
9. The tubes were centrifuged at 8000 x *g* for 30 min at room temperature.
10. The supernatant was discarded and the precipitate was resuspended with 70% ethanol at room temperature.
11. The tubes were centrifuged 10 min at 8000 x *g*. The ethanol was discarded and the precipitate dried.
12. The precipitate was dissolved in 1 mL of TE^3 buffer containing RNase 10 mg/mL and left for 1 h at 37 ºC.
13. An equal volume of chloroform/isoamyl alcohol (24:1) (CIA) was added.
14. The microtubes were gently mixed for 30 min and centrifuged at 6000 x *g* for 30 min at room temperature.
15. The supernatant was transferred to a new tube.
16. 0.6 vol of isopropanol were added and gently mixed allowing for the DNA to precipitate for 15 min at room temperature.
17. The DNA was recovered by centrifugation at 12000 x *g* for 30 min at 4 ºC.
18. DNA precipitate was resuspended in 1 mL TE and transferred to a clean tube and washed with 1 mL of 70% ethanol.
19. The microtubes were centrifuged 5min at 16000 x *g* and the ethanol discarded, followed by incubation for 20 min to evaporate the remains of ethanol.
20. 1 mL of TE was added and the DNA dissolved (gently heated if necessary).
21. An equal volume of Phenol:CIA (1:1) was added. The tubes were gently mixed and centrifuged 5 min at 16000 x *g.*
22. The upper layer was transferred to a new microtube and an equal volume of CIA was added. The microtubes were centrifuged 5 min at 16000 x *g.*
23. The upper layer was transferred to a new microtube. 1/10 of NaAc, pH 5.2 and 2.5 volumes of cold absolute ethanol were added to the transferred volume.

24. DNA was left to precipitate 16-18 h at -20 ºC and centrifuged 30 min at 16000 x *g* at 4ºC.
25. The precipitate was washed with 70% ethanol and centrifuged 5 min at 16000 x *g*.
26. The precipitate was dissolved in 200 μL of TE.
27. DNA presence was confirmed by loading 10 μL of DNA in a 1% agarose gel and stained with ethidium bromide (0.5 μg/mL).

[1] Solution I: 50 mM Glucose, 25 mM Tris-HCl, 10 mM EDTA; [2] Solution II: 0.2 N NaOH, 1% SDS, H_2O to a final volume of 100 mL; [3] TE: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA

2.6 Introduction of DNA *in E. coli*

2.6.1 Transformation

For transformation procedures, *E. coli* TOP10 competent cells (Invitrogen, Carlsbad, California, USA) were used and the protocol was performed as follows:

a) Preparation of competent cells:

1. TOP10 bacterial cells were grown overnight at 37 ºC in Luria-Bertani broth (LB, Merck, Germany).
2. 1 mL of the culture was centrifuged at 16000 x *g* for 3 min.
3. The precipitate was washed 3 times with 1 mL of sterile water.
4. The precipitate was ressuspended in 80 μL of sterile water.

b) Transformation protocol:

1. The cloning product was added[1] to competent cells.
2. The cells were electroporated using the program Ec3, 1 pulse, in a Bio-Rad Gene Pulser II electroporator (Bio-Rad, Richmond, CA, USA).
3. 500 μL of LB broth (LB, Merck, Germany) were immediately added to the Gene Pulser® cuvette (Bio-Rad, Richmond, CA, USA) and the suspension was transferred to a microtube.
4. The microtube was incubated in a shaker at 37 ºC for at least 1 h.

5. The cells were plated on LB agar medium (LB agar, Merck, Germany) with the respective selective marker(s)[2].

[1]Cloning product: 100 ng of Plasmid DNA; [2]Selective marker (s): nalidixic acid 8 μg/mL, ampicilin 100 μg/mL, ciprofloxacin 0,125 μg/mL, sulphamethoxazole 100 μg/mL.

2.6.2 Conjugation

For conjugation procedures, *E. coli* J53^{AZr} was used as recipient strain and the protocol was performed as follows:

1. Bacterial cells (donor and recipient strains) were grown overnight at 37 ºC in Luria-Bertani broth (LB, Merck, Germany).
2. 1 μL loop was plated in Tryptic Soy Agar (TSA, Merck, Germany) to verify the purity of the strain.
3. The donor and the recipient strains were plated on LB agar medium (LB agar, Merck, Germany) in a 5:1 ratio with the respective selective marker(s)[1] and left for 18 h at 37 ºC.
4. The transconjugants were selected and the resistance transfer verified.

[1]Selective marker (s): nalidixic acid 8 μg/mL, ampicilin 100 μg/mL, ciprofloxacin 0,125 μg/mL, sulphamethozaxol 100 μg/mL.

2.7 DNA hybridisation

2.7.1 Labeling of DNA probes – Radio labeling and DIG labeling

Radio labeling

DNA fragments to be used as probes were labeled with [$^{\alpha\text{-}32}$P]-α-CTP by random primer extension using a commercially available kit (Stratagene, UK) according to the manufacturer's instructions.

1. 15 μL of DNA[1], 8 μL of DNase-free water and 10 μL of primers, were mixed together in a screw-top microtube and boiled for 5 min.
1. **Note**: Buffer dCTP was used.
2. 10 μL of Buffer, 2.5 μL of ^{32}P and 1 μL of Klenow, were quickly added to the microtube to keep temperature hot. The microtube tube was placed in a lead jar at 37 °C for 15 min.
3. Unincorporated nucleotides were removed by passing the labeled DNA through a Sephadex column (Nick column, Pharmacia Bio-tech, Uppsala, Sweden). 320 μL of 0.1 M Tris-HCl buffer, pH 7.5, were added and moved to a collection tube. 430 μL of Tris-HCl buffer were added and the run-through collected.
4. The collected run-through was boiled for 6 min and added to the gel in the hybridisation tube and incubated for 3-5 h or overnight.

[1] 50 -100 ng of purified DNA

DIG labeling of a PCR product

1. 5 μL of PCR product[1] were added to 11 μL of H_2O.
2. The microtube was placed in boiling water for 10 min.
3. The microtube was then immediately placed on ice for 5 min.
4. 4 μL of Dig-High Prime (Roche Molecular Biochemicals, Indianapolis, USA) were added and gently shaken.
5. After a short spin, the reaction was incubated overnight (16-20 h) at 37 ºC.
6. The microtube was incubated at 65 ºC for 10 min to stop the reaction.
7. The reaction was stored at -20 ºC until further use.

[1] 1 - 50 ng of DNA

2.7.2 Hybridisation of Pulse Field Gel Electrophoresis gels

The hybridisation was used for the location of the genes of interest. This method was applied to Pulse Field Gel Electrophoresis gels (See section 2.11) .

1. The gel was placed on a sheet of filter paper and left at 50 °C at least overnight.
2. The gel was then soaked in water for at least 45 min or until fully hydrated and placed in denaturing solution[1] for 45 min and then transferred to neutralising solution[2] for 45 min.
3. The gel was placed in the hybridisation tube and 20 mL of pre-hybridisation solution[3] were added.
4. The tube was placed into the hybridisation oven at 65 °C overnight and the labeled probe was added.
5. After 16-18 h tube content was removed and the gel wrapped in cellophane, covered with a Kodak XRP-1 film and stored at -80 ºC for 2 days.
6. The film was developed 1-5 min in Kodak GBX developer, rinsed in distilled water for 30 sec and fixed in Kodak GBX fixer for 5 min. The film was rinsed again in distilled water for 30 sec and air-dried.

[1]Denaturing solution: 1.5 M NaCl; 0.5 M NaOH; [2]Neutralising solution: 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2, 1 mM EDTA. [3]Pre-hybridisation solution: 6 mL 20xSSC (3 M NaCl, 0.3 M Sodium Citrate, pH 7.0), 5X Denhardt's solution (0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) ficoll type 400, 0.1% (w/v) bovine serum albumin), 300 μL of denaturated salmon sperm DNA (100 mg/mL) and water up to a final volume of 20 mL.

2.7.3 Colony Blot

The colony blot technique was used to detect the presence of the genes of interest. The method was performed as follows:

1. Colonies to be screened were “tooth-picked” on to media, with a positive and negative control colony and incubated overnight at 37 ºC.

2. The colonies were transferred to a positively charged nylon membrane (Amersham Hybond^{TM}-N+, GE Healthcare, UK).
3. The membrane was placed colony side up on a Whatmann 3MM filter, presoaked for 5 min with 5% SDS.
4. The excess moisture was removed, onto Whatmann 3MM paper.
5. The filter was placed onto a Whatmann 3MM paper presoaked with denaturing solution[1] for 5 min.
6. The excess of moisture was removed and the filters placed carefully floating colony side up on neutralising solution[2] for 5 min.
7. After 5 min, the filter was submerged in the neutralising solution and the cellular debris was carefully washed from the filter.
8. The excess of moisture was removed onto Whatmann 3MM paper.
9. The filter was submerged in 6X SSC solution for 5 min and then the excess of moisture was removed onto Whatmann 3MM paper.
10. The DNA was fixed to the membrane by incubation at 80 ºC for at least 2 h.
11. The membranes were pre-hybridized with pre-hybridisation solution[3] at 65 ºC for 2 h and the probe was then added and left overnight (16-18 h).
12. The membranes were washed with 2X SSC for 30 min at 65 ºC and 1X SSC for 30 min at 65 ºC, wrapped in cellophane, covered with a Kodak XRP-1 film and stored at -80 ºC for 2 days.
13. The film was developed 1-5 min in Kodak GBX developer, rinsed in distilled water for 30 sec and fixed in Kodak GBX fixer for 5 min. The film was rinsed again in distilled water for 30 sec and air-dried.

[1] Denaturing solution: 1.5 M NaCl; 0.5 M NaOH; [2]Neutralising solution: 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2, 1 mM EDTA. [3]Pre-hybridisation solution: 6 mL 20xSSC (3 M NaCl, 0.3 M Sodium Citrate, pH 7.0), 5X Denhardt's solution (0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) ficoll type 400, 0.1% (w/v) bovine serum albumin), 300 μL of denaturated salmon sperm DNA (100 mg/mL) and water up to a final volume of 20 mL.

2.8 Capture of DNA using Dynabeads M-280 Streptavidin system

To determine flanking sequences of a gene in the genome, a two-step PCR reaction technique can be applied using a biotinylated primer plus degenerate primers for amplification. Amplified biotinylated DNA fragments were isolated with a Dynabeads M-280 Streptavidin system (Sorensen *et al.* 1993). All PCR reactions were carried out using the PCR protocol outlined in section 2.3 but 42 cycles were employed for the first amplification. Genomic DNA was used as template in the first PCR reaction, whereas an aliquot from the first PCR reaction was used as a DNA template for the second PCR reaction.

1. PCR reactions with biotinylated primers designed for the target gene (Primer Rv and Fw) and random primers (R1, R2, R3, R4) were performed to a final volume of 50 μL as follows:

PCR mastermix	25μL	25μL	25 μL	25μL
Primer Rv or Fw	1μL	1μL	1μL	1μL
R1	1μL	-	-	-
R2	-	1μL	-	-
R3	-	-	1μL	-
R4	-	-	-	1μL
DNA	1μL	1μL	1μL	1μL
H_2O	22μL	22μL	22μL	22μL

PCR mastermix contained: 1X PCR buffer, 3 mM $MgCl_2$, 5% dimethylsulfoxide, 200 μM each nucleotide, 0.3 pmol/μL of each primer, 1 U of *Taq* polymerase, and 1 μL of heat-denatured cells or 50 -100 ng of purified DNA.

2. PCR amplifications were performed using the following program:

Initial Denaturation	95 ºC for 10 min
Denaturation	95 ºC for 1 min
Annealing	45 ºC for 1 min
Elongation	68 ºC for 1 min
Extension	68 ºC for 5 min

3. The dynabeads were washed three times with 2X wash buffer (TE).

Note: Beads stock was at 10 mg/mL. 200 μg of beads per reaction were needed and were ressuspended in twice of the initial volume.

4. 40 μL of PCR product were mixed with 40 μL of Dynabeads (containing 200 μg) and incubated for a minimum of 15 min at room temperature.
5. The beads were washed with 1X wash buffer and resuspended in 10 μL of 1X wash buffer.
6. 5 μL of beads were used for the next PCR reaction with flanking primers (NF primer).

PCR mastermix	25μL	25μL	25 μL	25μL
Primer Rv or Fw	1μL	1μL	1μL	1μL
NF primer	1μL	1μL	1μL	1μL
Beads	5μL	5μL	5μL	5μL
H_2O	18μL	18μL	18μL	18μL

PCR mastermix contained: 1X PCR buffer, 3 mM $MgCl_2$, 5% dimethylsulfoxide, 200 μM each nucleotide, 0.3 pmol/μL of each primer, 1 U of *Taq* polymerase, and 1 μL of heat-denatured cells or 50 -100 ng of purified DNA.

7. PCR amplifications were performed using the program described below:

Initial Denaturation	95 ºC for 10 min
Denaturation	95 ºC for 1 min
Annealing	58 ºC for 1 min
Elongation	68 ºC for 2 min
Extension	68 ºC for 5 min

8. PCR products were separated by electrophoresis in a 1% agarose gel in 1X TBE buffer (TBE Buffer 10X: 0.89 M Tris Borate pH 8.3).

2.9 DNA Digestion

DNA was digested with restriction endonucleases as follows:

DNA	200-1000 ng
10X enzyme buffer	1 μL
Restriction enzyme	1 μL

Sterile, distilled water was added to a final volume of 10 μL and the reaction was incubated at enzymes respective temperature.

When restricted DNA was to be visualized on an agarose gel without further treatment, the reaction was terminated by the addition of 3 μL stop mix (40% (w/v) sucrose, 0.2M EDTA pH 8.0 and 0.15% (w/v) bromophenol blue). Otherwise reactions were terminated using a phenol chloroform wash or by heating at the appropriate temperature for 10 min

Where partial digestions were required, preliminary reactions were performed in order to determine optimal reaction conditions. Serial dilutions of the enzymes were prepared and samples were withdrawn at set time intervals and the products analysed by agarose gel electrophoresis. The restriction was then repeated using those conditions that produced the desired partial digestion, in the preliminary reaction.

2.10 DNA Ligation

Ligation of DNA was performed with T4 DNA ligase in a final reaction volume of 10 μL. For subcloning experiments, a 3:1 molar ratio of insert DNA to vector was used. Ligation reactions were incubated overnight at 20 ºC. The ligation mix was heated at 70 ºC for 10 min and micro-dialysed on filter discs (0.025 μm pore; Milipore, UK) for 30 min prior to electroporation.

2.11 Pulse Field Gel Electrophoresis (PFGE)

Pulse field gel electrophoresis was used to acess gene location and bacterial typing.

1. Strains were grown overnight on agar plates at 37 ºC.
2. A 10 μL loop of cells suspended in 3 mL of saline were harvested in a bijou tube.
3. The $OD_{(Abs:600nm)}$ of each strain was measured. The volume of cells was adjusted to the equivalent of 300 μL at $OD_{(Abs:600nm)}$ of 1.5 following the formula: 1.5/measured OD x 300 μL.
4. The cells were centrifuged at 16000 x *g* for 30 sec and the supernatant was removed.
5. The cells were resuspended in 300 μL of normal saline and placed in a dry block at 50 ºC.
6. 2-3 drops of lysozyme at the concentration 25 mg/mL were added, immediately followed by 300 μL of pre-warmed (50 ºC) 2% low melting point agarose (Sigma, Germany) and gently mixed with a pipette.
7. The mix was quickly loaded into the PFGE plug moulds and after 15-20 min setting time, the plugs were removed from the plug moulds and each set of 5 placed in 2 mL of lysis buffer[1] in an individual well of a 24 well plate. The lysis buffer was supplemented with 80 μL of lysozyme (25 mg/mL) per well and incubated for 1.5 h at 37 ºC.
8. The plugs were washed with 2 mL of 1X TE buffer for 30 min at 37 ºC.
9. The TE buffer was removed and replaced by 2 mL of proteolysis buffer[2] containing 20 μL of 10 mg/mL Proteinase K.
10. The plugs were incubated at 50 ºC for 18 h.
11. After this the plugs were washed 5X30 min in 1X TE buffer. At this stage the plugs can be stored for 3-6 months. Plug digestion is carried out in individual wells of a 48 well plate. Up to 2 plugs can be digested in a single well.

S1 digestion of agarose plugs:

1. The plugs were washed in 1:10 TE buffer with gentle shaking for 30 min at room temperature.
2. The plugs were then washed 15 min with 1X S1 buffer or left overnight at 4 ºC.
3. The buffer was then removed and 1 μL of 1:4000 *S1* enzyme (NewEngland Biolabs, UK) was added directly to the plug. The plugs were incubated at 37 ºC for 45 min precisely. The digestion is stopped by adding 100 μL of ES buffer for 5 min at 4ºC.
4. The plug was suspended in TE buffer and loaded onto the gel.

*Sma*I digestion of agarose plugs:

1. The plugs were washed in 1:10 TE buffer with gentle shaking for 30 min at room temperature.
2. The plugs were then washed and left overnight at 4 ºC with 1X *Sma*I buffer.
3. The buffer was then removed and 1 μL of *Sma*I enzyme (NewEngland Biolabs, UK) was added directly to the plug. The plugs were incubated at 37 ºC for 3 h.
4. The plug was suspended in TE buffer and loaded onto the gel.

Agarose gel and electrophoresis conditions:

1. A 1% agarose gel was prepared in 0.5% TBE buffer and 20 μL ethidium bromide (0.5 μg/mL) were added.
2. The gel was removed from the tray and placed in the electrophoresis tank previously washed with 70% ethanol.
3. The DNA was separated in the Chef DR111 System (Bio-Rad, Richmond, CA USA) with the following conditions: Bio-Rad Cooling module was set at 14 ºC, an initial pulse 5.0, final pulse 45.0, running time 20 h, 6.0 volts and an angle 120.

[1]Lysis buffer: 10 mM Tris-HCl, pH 7.2, 50 mM NaCl, 0.2% sodium deoxycholate, 0.5% N-lauroylsarcosine; [2]Proteolysis Buffer: 100 mM EDTA, pH 8, 0.2% sodium deoxycholate, 1% lauroylsarcosine; TE Buffer 1x: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA; ES Buffer 0.5 M EDTA, pH 8, 1% N-lauroylsarcosine; TBE Buffer 10x: 0.89 M Tris Borate pH 8.3.

3. Chapter 1 – IS*CR1* prevalence in *Escherichia coli* clinical isolates

3.1 Introduction

Escherichia coli, a member of the Enterobacteriaceae family, is the main aerobic commensal bacterial species in the bowel flora yet, in the urinary tract or in blood, it can become pathogenic. In fact, it is the leading Gram-negative pathogen causing nosocomial and community acquired infections. Since *E. coli* easily acquires resistance and is commonly found in many different animal species, its antimicrobial resistance profile is well documented worldwide (Erb *et al.* 2007; von Baum and Marre 2005). It is frequently resistant to aminopenicillins, such as amoxicillin or ampicillin and narrow-spectrum cephalosporins (Tenover 2006). Multiresistant isolates are becoming a common presence within the clinical environments, mainly because bacteria have an impressive capacity to acquire new genes and to rearrange its genetic information (Toleman *et al.* 2006b). For instance, plasmids started being acquired from environmental bacteria and throughout time have been combining with other elements such as transposons, integrons, insertion sequences (IS) and more recently with insertion sequence common regions, or IS*CR* elements (Walsh 2006). Most of these elements carry antibiotic resistance, whereas other are involved in the mobilization of such genes. In *E. coli* resistance is now becoming addressed to all antibiotic spectrums, for instance β-lactam, quinolones, aminoglycosides. The most common mechanism of resistance to β-lactam antibiotics is the production of β-lactamases. These enzymes are encoded by genes that evolve rapidly, thus constituting a group characterized by high levels of molecular diversity. CTX-M β-lactamases are plasmid-mediated ESBLs and confer high-level resistance to cefotaxime, ceftriaxone and aztreonam and are well inhibited by clavulanate and tazobactam (Karim *et al.* 2001). The $bla_{\text{CTX-M}}$ genes encoding these β-lactamases are involved in this resistance, with a increasing predominance of $bla_{\text{CTX-M-15}}$ (Eckert *et al.* 2004b). The $bla_{\text{CTX-M-15}}$ gene is typically associated with IS*Ecp1* as part of a transposition unit (Walsh 2006). In 2006, Machado *et al*. investigated the presence of IS*CR1* in 17 Portuguese ESBL positive clinical isolates; however, IS*CR1* could not be detected (Machado *et al.* 2006). Unfortunately, this study was naturally

biased and did not reflect the true prevalence of ESBL or antibiotic resistance gene carriage. Considering the results obtained by these authors, our aim was to evaluate the prevalence and occurrence of IS*CR1* elements, integrons and β-lactamase genes in a non-biased population. For that, 210 non-biased *E. coli* isolates were collected from three separate patient-cohorts: day care (DC), emergency room (ER), and long term inpatients (IP) in the hospital Infante D. Pedro, EPE, Aveiro.

3.2 Results and Discussion

3.2.1 Isolates background

A total of 210 *E. coli* strains were collected from different biological products, between May 1st, 2006 and November 30th, 2006 at Hospital Infante D. Pedro, EPE, Portugal. The population analysed was non-repetitive and only single isolate/patient was included in the study. Species identification was performed with VITEK2 Compact and Advanced Expert System (AES) (bioMérieux, Marcy L´Étoile, France). Thirty-six isolates were collected from the DC, one hundred and twenty four collected from the ER and fifty from IP. Figure 18 shows the range of age of the patients from whom the strains were isolated versus the number of isolates (1 from each patient) in each interval. As expected, a higher number of isolates were retrieved from the elderly (>70) and infants (<1) groups.

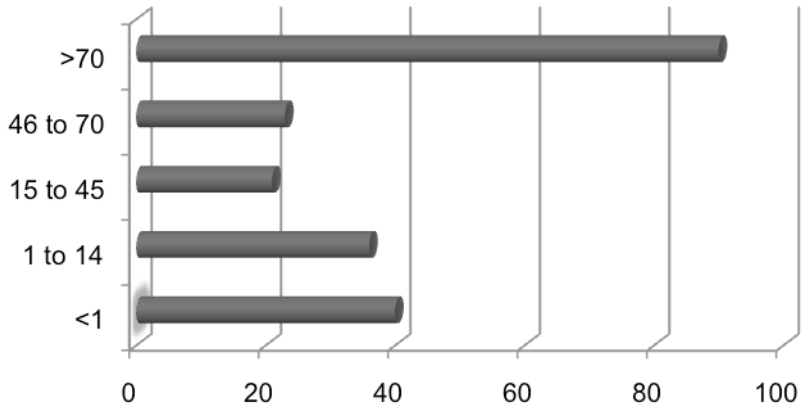


Figure 18 – Patients ages interval is represented in the vertical axis and horizontal axis represents the number of patients from whom the *E. coli* isolates were collected.

The isolates were collected from different clinical specimens as shown in Table 3. Urine yielded the higher number of isolates in the 3 cohorts. The result was not surprising since *E. coli* is one of the pathogens frequently associated to urinary tract infections.

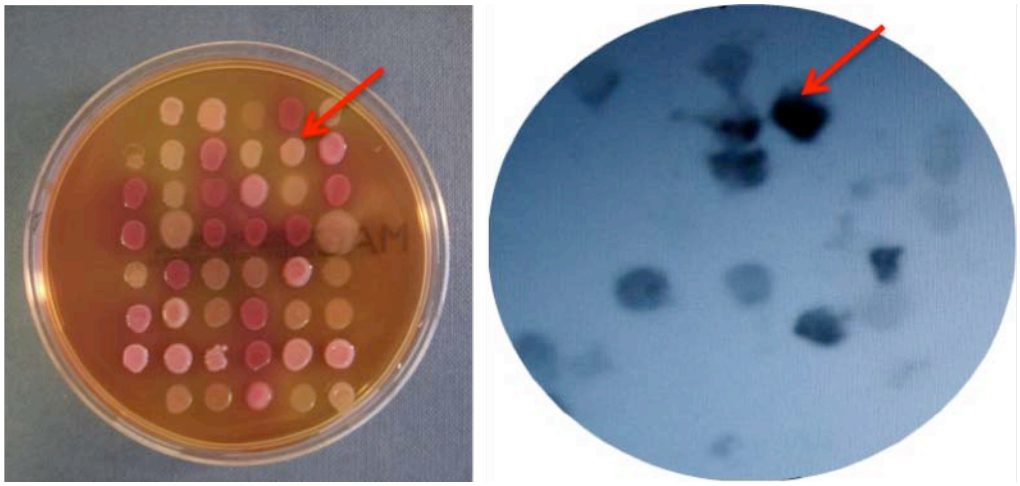
3.2.2 Antimicrobial susceptibility testing

Susceptibilities were determined, according to guidelines of CLSI standards (CLSI 2006). It has been reported that resistance to antibiotics, such as ampicillin and tetracycline, may be linked to the time that they are available in the clinical practice (White *et al.* 2001). Thus, in the present study, it was also found that ampicillin was the β-lactam where higher levels of resistance (64%) were observed. As representative of the carbapenems, meropenem is often a choice for systemic infections treatment. However, if until recently reports of carbapenem resistant Enterobacteriaceae were rare, now they are becoming frequent, thus compromising carbapenems efficacy (Nadkarni *et al.* 2009; Tokatlidou *et al.* 2008). All isolates analysed were sensitive to meropenem concurring with other studies performed in Portugal (Mendonça *et al.* 2007). Among cephalosporins, first generation cephalotin, exhibited higher percentage of resistance (31%) when compared with the fourth generation compounds represented by cefepime (11%). Twenty-five isolates (12%) were simultaneously resistant to cefotaxime and ceftazidime. There was no discernable difference between levofloxacin and norfloxacin. Moreover, co-resistance to quinolones and cephalosporins was observed in 41 isolates (20%) concurring with the studies by Morosini *et al.* that co-resistance observed is a key factor in the maintenance and dispersion of these isolates (Morosini *et al.* 2006). Since this is a class of antibiotics commonly used to treat urinary tract infections, along with β-lactams and macrolides, the increasing level of resistance exhibited by an increasing number of isolates tends to render them ineffective in the clinical treatment. Resistance observed among the aminoglicosides was higher to gentamicin (19%) when compared to amikacin

(1%). Morosini and co-workers (2006) obtained similar results of amikacin efficacy in a study with ESBL-producing Enterobacteriaceae isolates.

3.2.3 IS*CR1* detection by colony blotting

Hybridisation experiments were conducted to evaluate the occurrence of IS*CR1* using the colony blot methodology described in Material and Methods section 2.7.3.



A) **B)**

Figure 19 – A) *E. coli* isolates grown in MacConkey agar (bioMérieux, France); B) A positive result was revealed by autoradiography of the blotted colonies using the IS*CR1* probe. Arrows indicate an example of a positive result.

IS*CR1* positive colonies were subsequently confirmed by PCR followed by nucleotide sequence confirmation. The amplicons were separated in a 1% agarose gel (Figure 20).

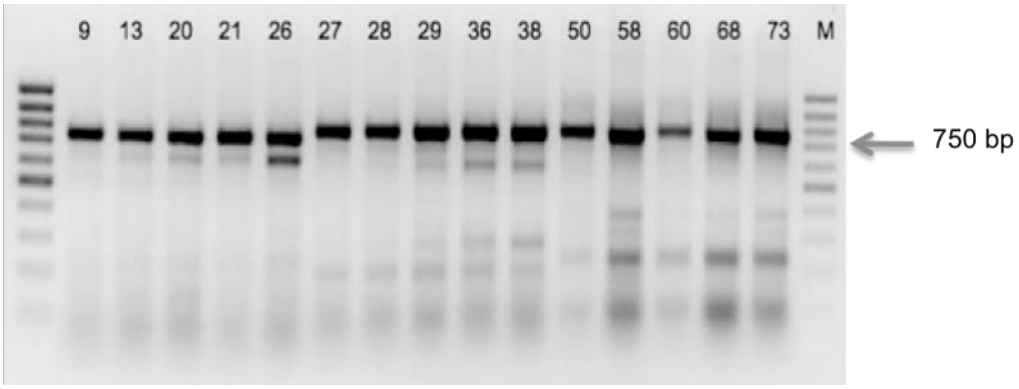


Figure 20 – IS*CR1* amplicons of the *E. coli* positive colonies, separated in a 1% agarose gel electrophoresis. Primers used were previously described in Material and Methods section 2.3; M- GeneRuler 100 bp DNA Ladder (MBI Fermentas, Vilnius, Lithuania).

The presence of IS*CR1* was detected in 42 isolates (20%) (Table 3) which can be considered a high occurrence of such mobile genetic determinant. Moreover, they were found evenly distributed between DC, ER and IP. All IS*CR1* positive strains harbored a class 1 integron, which reinforces the linkage between these two structures. Interesting the study by Machado *et al*. reported that no IS*CR1* (CR1) elements were found from strains in 2005-2006, which suggests that the advent and dissemination of IS*CR1* is a recent event in Portugal. Accordingly, this is the first description of IS*CR1* elements in *E. coli* isolates from Portugal. Interestingly, IS*CR1* elements were detected in strains with low resistance levels suggesting that these bacteria maintain “genetic tool kits” for the acquisition of antibiotic resistance genes (Bennett 2008).

3.2.4 Class 1 integrons screening

Screening for class 1 integrons was performed in two steps. First the *int1* gene was screened by PCR and the positives strains were subsequently submitted to a new PCR to amplify the corresponding variable regions. Amplification conditions and primers were used as described in Material and Methods section 2.3.

intI1 gene was detected in 20% of the isolates. A total of 10 gene cassettes occurring in 8 different cassette arrays were identified – where the variants of the *aadA* and *dfr* genes were the most frequently found (Table 3). *aadA* gene variants confer resistance to streptomycin and spectomycin and represent 30% of the total gene cassettes found among the isolates.

Trimethoprim resistance is associated with *dfr* genes, usually inserted in integrons (Machado *et al.* 2005). In the present study *dfr* genes represent 50% of the gene cassettes detected. However, the presence of class 1 integrons could not be detected in all co-trimoxazole resistant isolates (26%). These results may suggest that sulphamethoxazole resistance may be due to the presence of other *sul* genes apart from *sul*1.

The results obtained in both the β-lactamase genes screening and class 1 integron detection suggest that the dissemination of these resistance determinants is a worldwide phenomenon. For instance, the array [bla_{OXA-30} –*aadA1*] was described in 2003 (AY224185) in an *E. coli* clinical isolate from France. Additionally, the array [*dfrA17-aadA5*] has previously been described in *A. baumannii* (EF015498), *Enterobacter cloacae* (EF571855) and *P. aeruginosa* (DQ838665). When described in *E. coli* this array was found in isolates from animal (DQ663488) as well as the clinical sector (AB194702 and FJ0011847) and scattered all over the world in countries so distant as China, Turkey and the Netherlands. The rapid dissemination of these structures between different countries may be explained by the society behavior. Nowadays, traveling is easier and faster, leading people to act as vehicles to these structures, by transportation of bacteria that carry them, from one point to another (Hawkey and Jones 2009).

Table 3 – Characterization of *E. coli* isolates concerning demographic data, integrons content and *bla* genotypes.

		Day care	Emergency Room	Inpatients (Nosocomial)
Total number of isolates		36	124	50
Range of ages		<1 to >70	<1 to >80	3 to >80
Biological Products	**Urine**	31	84	33
	Blood	0	18	2
	Other[a)]	5	22	15
***intI1* gene**		8 (22%)	26 (20%)	8 (16%)
Putative gene cassettes		*dfrA25* *dfrA12-aadA2* *dfrA17-aadA5* *dfrA1-aadA1*	bla_{OXA-30}–*aadA1* *dfrA1-aadA1* *spec-aadA1* *dfrA4*	*dfrA1-aadA1* *aadA1*
IS*CR1*		8 (22%)	26 (20%)	8 (16%)
β-lactamase content	bla_{TEM-1}	17(47%)	99 (80%)	18 (36%)
	bla_{OXA-30}	0	8 (6%)	17 (34%)
	$bla_{CTX-M-15}$	5 (14%)	15 (12%)	5 (10%)

a) Other- Sputum, swab wounds, pus;

3.2.4 β-lactamase genes content

A first screening of β-lactamase genes content was performed in the VITEK2 Compact and AES (BioMérieux, Marcy L´Étoile, France). Additionally, and to confirm VITEK2 system results, the presence of β-lactamase genes belonging TEM, CTX-M, OXA groups was screened by PCR, and the nucleotide sequence of the amplicons was determined. Amplification conditions and primers used were previously described in Material and Methods section 2.3.

PCR based methods are not yet implemented in the routine of the majority of the hospitals in the country and Hospital Infante D. Pedro is not an exception. The use

of Etest strips is therefore an important tool to be used for the rapid detection of ESBL-producing strains. Etest ESBL strips with cefotaxime/ cefotaxime + clavulanic acid (CT/CTL), ceftazidime/ ceftazidime + clavulanic acid (TZ/TZL) and cefepime/ cefepime + clavulanic acid (PM/PML) were used according to the manufacturer's instructions (AB bioMérieux, Solna, Sweden) to detect ESBL production. The comparison between the results obtained from the VITEK2 Compact and Etest ESBL strips, and further confirmed by PCR, revealed that the two methods provided similar results. 43% of the isolates were positive for the presence of an ESBL, noteworthy 10% of these were isolated from biological products from the Pediatrics ward (inpatients). This result is of foremost concern, highlighting the growing number of strains carrying ESBLs in the pediatrics population. The spread of resistance determinants such as ESBL poses a major problem for the treatment of these patients since only a few of the available antibiotics can be used in the pediatric patients, which narrows the treatment options.

PCR results obtained for each β-lactamase gene group tested revealed that *bla*$_{TEM}$ was present in 64% of the isolates studied and both *bla*$_{CTX-M}$ and *bla*$_{OXA}$ were detected in 12% of the isolates. Nucleotide sequence analysis revealed that all the isolates that were positive for the presence of *bla*$_{TEM}$, possessed the *bla*$_{TEM-1}$ variant. Moreover, all were ampicillin resistant, suggesting that this gene might be responsible for the resistance observed. All the isolates positive for the presence of *bla*$_{CTX-M}$ were resistant to cefotaxime. A study conducted in Spain by Valverde and colleagues showed that strains carrying *bla*$_{CTX-M}$ have a higher tendency to be MDR strains, when compared to strains carrying other type of ESBLs (Valverde *et al.* 2004). For instance, strains producing *bla*$_{CTX-M}$ exhibit higher levels of resistance to tetracyclines and ciprofloxacin, when compared to strains producing *bla*$_{TEM-4}$ and *bla*$_{SHV-12}$. In the present study, 75 % of *bla*$_{CTX-M}$ producing strains were also resistant to quinolones and 42% to tetracycline.

The genetic environment of *bla*$_{CTX-M}$ genes found among the population being studied, was investigated. Nucleotide sequence analysis identified *bla*$_{CTX-M-15}$ as the variant present in the isolates positive for the presence of this gene. These findings are consistent with those reported by other authors, regarding the dissemination of *bla*$_{CTX-M-15}$ in Portugal, where this gene is the predominant variant

(Mendonça *et al.* 2007). Interestingly, fifteen *bla*$_{CTX-M-15}$ positive isolates were recovered from the emergency room from patients with no previous clinical records, and were thus considered to be from the community background, suggesting its presence in other reservoirs as also reported by other authors (Mendonça *et al.* 2007). Several studies worldwide report *bla*$_{CTX-M}$ dissemination in both clinical and community environments, and among different Gram-negative species (Prats *et al.* 2003). Although the isolates positive for the presence of *bla*$_{CTX-M}$ were resistant to cefotaxime, not all cefotaxime resistant isolates (18%) possess the *bla*$_{CTX-M}$ gene. In these cases, the presence of another broad range β-lactamase should be expected, namely *bla*$_{DHA-1}$, a plasmid mediated AmpC with hydrolytic activity against third generation cephalosporins (Yan *et al.* 2002)

Nucleotide sequence analysis identified *bla*$_{OXA-30}$ in strains positive for *bla*$_{OXA}$, which has a special hydrolytic activity against cloxacillin and also hydrolyzes cefepime (Dubois *et al.* 2003). Thus, the results obtained may explain the resistance exhibited by these positive isolates. Nucleotide sequence analysis also revealed that *bla*$_{OXA-30}$ gene was located within an integrants in eight isolates. The genetic location of *bla*$_{OXA-30}$ genes in mobile genetic elements such as integrons may potentiate their dissemination among other isolates (Dubois *et al.* 2003).

3.2.5 General Conclusion

The vast majority of the isolates included in this study were isolated from urine and from the two cohorts Day Care and Emergency Room. IS*CR1* was found evenly distributed among Day Care, Emergency Room and inpatients. Integrons were detected in the three cohorts. However, seven different arrays were found in isolates from Day Care and Emergency Room and only two arrays were detected in inpatients. *bla*$_{TEM}$ was the predominant *bla* gene in the three cohorts.

The presence of mobile genetic elements such as the IS*CR*s in these isolates is preoccupant since it can enhance the dissemination of resistance determinants under favorable circumstances.

4. Chapter 2 – First description of *Klebsiella pneumoniae* clinical isolates carrying both *qnr*A and *qnr*B genes in Portugal

4.1 Introduction

Multi-drug resistant (MDR) organisms continue to represent a significant challenge in the management of hospitalized patients with infections. Such infections are often opportunistic and are associated with hospitalization, and include pneumonia, urinary tract and wound infections and neonatal meningitis. Organisms such as MDR *K. pneumoniae* are common nosocomial pathogens causing severe morbidity and mortality (Melano *et al.* 2003). Additionally, Klebsiellae survive longer than other enteric bacteria on hands and environmental surfaces, facilitating cross-infection within hospitals (Paterson and Bonomo 2005). For instance, even prior to the advent of ESBLs, large multiresistance plasmids were more common in *Klebsiella spp*. than in *E. coli,* contributing to a fast dissemination of antibiotic resistance genes. Moreover, most *K. pneumoniae* isolates have a chromosomally encoded SHV-1 β-lactamase (Melano *et al.* 2003). Plasmid-mediated quinolone resistance encoded by *qnr*A, *qnr*B or *qnr*S genes has been reported worldwide (Nordmann and Poirel 2005). *qnr*A, was the first *qnr* gene described in a *K. pneumoniae* in by Martinez *et al.* (1998). Subsequently, it has been shown to be widely spread all over the world, as part of a complex *sul1*-type integron containing IS*CR1*(Nordmann and Poirel 2005). *qnrB* and *qnrS* have also been reported in many countries from Europe, Asia and the United States (Nordmann and Poirel 2005). Dissemination of plasmids carrying these genes can potentiate the rapid development of higher-level quinolone resistance in bacterial strains currently classified as susceptible (Robicsek *et al.* 2005a). Moreover, transferrable *qnr* genes are usually carried by conjugative plasmids (50 to 180kb) that often encode ESBLs or AmpC-type β-lactamases (Lascols *et al.* 2008). In fact, epidemiological studies of the distribution of *qnr* determinants show that *qnr* positive strains frequently express ESBLs (Garnier *et al.* 2006). Cephalosporins, such as cefotaxime and ceftriaxone, are commonly used to treat Klebsiellae infections, however multiple MDR strains may limit the antibiotic choice.

Herein a MDR *K. pneumoniae* population, collected from inpatients from the Intensive Care Unit was characterized and the occurrence of IS*CR1* associated with other antibiotic resistance genetic determinants was investigated.

4.2 Results and Discussion

4.2.1 Clinical context of *K. pneumoniae* recovery

The population of *K. pneumoniae* collected was constituted by MDR isolates associated with infection. The patients infected were hospitalized in the Intensive Care Unit (ICU). The isolates were retrieved from different biological products including sputum (10 isolates), blood (2 isolates), pus (4 isolates), urine (3 isolates) and catheter (1 isolate) (Figure 21). As expected, the biological product from which a higher number of isolates was collected was the sputum. These results may be explained by the fact that the majority of the patients hospitalized in this ward are usually diagnosed with respiratory tract infections being *K. pneumoniae* one of the major pathogens associated with it.

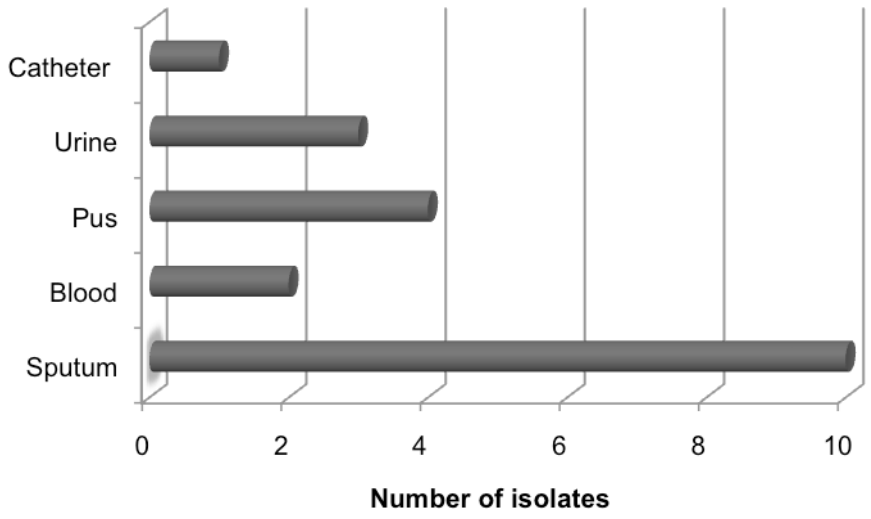


Figure 21 – Biological products and number of *K. pneumoniae* isolates selected for the present study.

The ages of the inpatients ranged between 43 and 91 years. The advanced age of most of the patients can be critical for the treatment, since the patients hospitalized in the ICU are under heavy doses of medication, thus being very debilitated and with a compromised immune system. Therefore an infection caused by a pathogen such as MDR *K. pneumoniae* represents an enormous threat to the success of the treatment.

4.2.2 Antimicrobial susceptibility testing

Susceptibilities were determined, according to guidelines of CLSI standards (CLSI 2006). Antibiotic susceptibilities were tested to 21 antibiotics belonging to the following classes: β-lactams including the penicillins (aminopenicillins, carboxypenicillins and ureidopenicillins), cephalosporins (first, second, third and fourth generations), monobactams and carbapenems. Additionally, another four classes of antibiotics were tested: tetracyclines, fluoroquinolones, aminoglycosides, and the combination sulphamethoxazole/trimethoprim.

In general, the pattern of resistance was very similar among the different isolates. Strains that were resistant to at least three different classes of antibiotics were considered as MDR. β-lactam, fluoroquinolones and the combination sulphametoxazole/trimethoprim were the three different classes of antibiotics, to which higher resistance was observed. All the isolates were sensitive to the carbapenems, imipenem and meropenem.

Quinolones resistance profile of the 21 isolates revealed that all of them were resistant to nalidixic acid (≥ 32 µg/mL) and the fluoroquinolone ciprofloxacin (≥ 4 µg/mL). Nineteen isolates were intermediate resistant (8 µg/mL) and two were resistant (≥ 16 µg/mL) to norfloxacin (Table 4). These results indicate that the use of this class of antibiotics may be seriously compromised in a short-term period. In the clinical practice, intermediate resistant isolates to a given antibiotic should be reported to the clinician, as resistant isolates. These isolates exhibit *in vitro* a reduced susceptibility to the antibiotic that can easily lead to a resistant phenotype *in vivo*. Therefore, the nineteen strains with reduced susceptibility to fourth

generation quinolones should be reported as resistant, alerting the clinicians to a vigilant use of this antibiotic.

4.2.3 IS*CR,* β-lactamase genes content and class 1 integrons

Beta-lactamase genes screening was performed by PCR using the primers and conditions described in the Material and Methods section 2.3. The results revealed that *bla*$_{TEM}$ was found in 38% of the isolates and *bla*$_{SHV}$ in 86%, which may explain the resistance to penicillins, namely to ampicillin, exhibited by the isolates. *bla*$_{OXA}$ was present in 38% of the isolates and was found to be part of the In*37* array in all of the isolates. Nucleotide sequence analysis of the amplicons identified a *bla*$_{OXA-30,}$ which may be contributing to the amoxicillin resistance observed. Interestingly, although a genetic linkage between *bla*$_{CTX-M}$ and *aac(6')-Ib-cr* has been reported before (Cordeiro *et al.* 2008; Kim *et al.* 2009b; Machado *et al.* 2006), *bla*$_{CTX-M}$ was not detected. However, all cephalosporin resistant isolates harboured *qnr* genes, thus suggesting a linkage between the presence of *qnr* and the resistance to cephalosporins as previously reported (Cavaco *et al.* 2007b). Strains Kp4 and Kp5, although negative for the presence of *bla*$_{CTX-M}$ genes, harboured *qnr*A, *qnr*B and *bla*$_{DHA-1}$ encoding a plasmidic AmpC-type β-lactamase that confers resistance to cephamycins and extended-spectrum cephalosporins, which would explain the resistance to cephalosporins exhibited by these isolates. Moreover, *int1* was detected in 81% of the isolates and 38% carried more than one integron. A total of 10 gene cassettes occurring in 3 different cassette arrays were identified where the variants *aadA* and *dfrA* genes were the most frequently found (Figure 22). The complex integron In*37* [*aac(6')-Ib-cr*, *bla*$_{OXA-30}$, *catB3, arr-3*]:IS*CR1*:*qnr*A1 was found in 8 isolates, with the same structure previously described in an *E. coli* isolate from China (accession number AY259086).

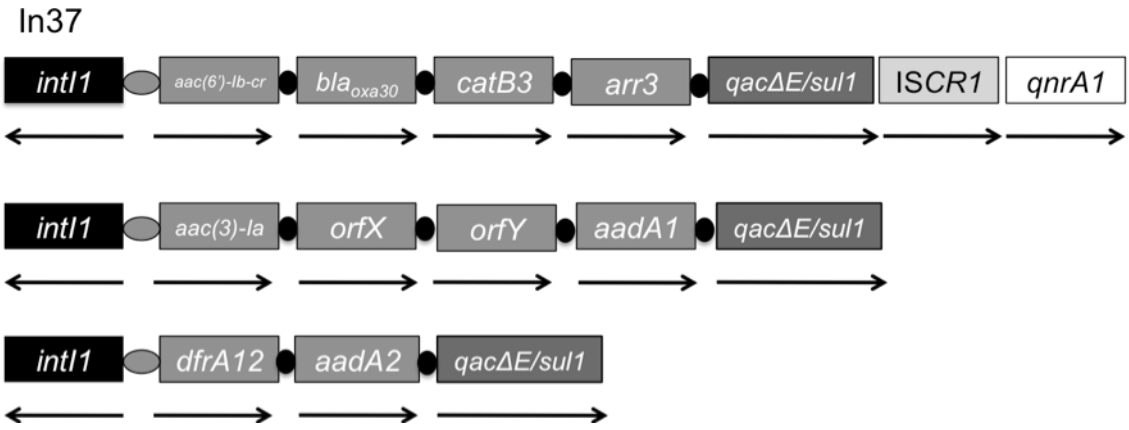


Figure 22 – Schematic representation of a class 1 integron found among the *K. pneumoniae* population under study. Light grey box- antibiotic resistance genes cassettes; black box- integrase gene, silver box, IS*CR1*; white box, *qnrA1*. Arrows indicate the direction of transcription. Solid black circles represent 59-base elements and grey ellipses represent the *att*I1 site of the integron.

The results showed also a high association between *aac(6')-Ib-cr* , *bla*$_{\text{OXA-30,}}$ class 1 integron, IS*CR1* and *qnrA* as previously reported in other studies, suggesting the dissemination of this structure in some parts of the Globe (Kim *et al.* 2009b; Wang *et al.* 2003). The gene cassette array [*aac(3)Ia-orfX-orfY-aadA1*] was found in 2 isolates. This array has been described in 2001, in an *A. baumannii* clinical strain from Italy (Accession number AJ310480). The same array was also found in *A. baumannii* isolates recovered in the Hospital Infante D. Pedro, EPE (see Chapter 4 of this thesis). The third class 1 integron gene cassette array found [*dfrA12-aadA2*] was found in fifteen isolates and has also already been described in a *Salmonella enterica* environmental strain from Japan (Accession number AB365868). Again, these results highlight the dissemination of these resistance structures worldwide, in different backgrounds and between different species. All the three class 1 integrons arrays found harboured gene cassettes conferring resistance to aminoglycosides, namely *aadA* and *aac* variants, which may explain the resistance exhibited by the isolates to this class of antibiotics.

An attempt to identify the reservoirs of the class 1 integron found on an active transposon, *K. pneumoniae* isolates collected in two distinct geographical regions were screened.

The rationale behind the screen of these isolates was the fact that Tn*5090* an active transposon, was originally found in *Klebsiella spp*. and this species are common soil bacteria that live in close association with other environmental bacteria.

81% (17/21) of the *K. pneumoniae* isolates from Portugal and 88% (32/36) of isolates from Libya were positive for *tniC* by colony blotting as described in section 2.7.3. Isolates from each country were selected for further study and were found to harbour the variable region consisting of the identical gene cassettes *dhfr*, *orf*D and *qacE* found in Tn*5090* (Figure 23).

Figure 23 – Schematic representation of a class 1 integron found among the *K. pneumoniae* population under study. Light grey box- antibiotic resistance genes cassettes; black box- integrase gene, silver box- resistance to quaternary compounds and a transposition gene. Arrows indicate the direction of transcription. Solid black circles represent 59-base elements and grey ellipses represent the *att*I1 site of the integron.

S1 restriction analysis of a subset of *tni*C positive strains indicated that the *tni*C gene was found on large plasmids of 400kb and 500kb in the isolates collected in Portugal (2 isolates tested) (Figure 24: 1) and 100kb to 500kb in isolates from Libya (7 isolates tested) (Figure 24: 2). Most of the isolates also carried the *tni*C gene on more than one plasmid and there was also evidence of the *tni*C gene being chromosomally located in a small number of bacteria.

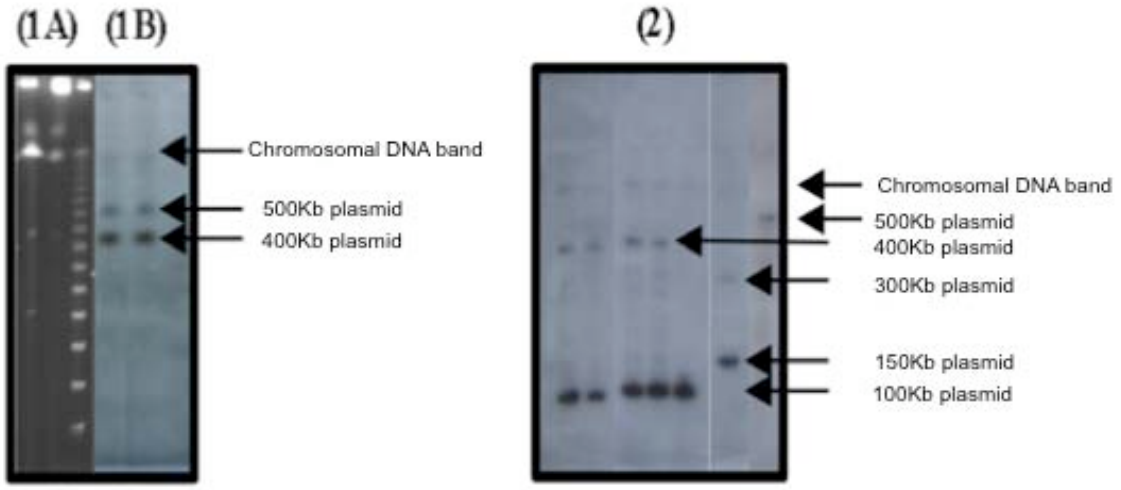


Figure 24 – 1) Pulse field gel electrophoresis analysis of partially digested DNA with S1 endonuclease of two *K. pneumoniae* strains isolated in Aveiro, Portugal. Lane 1 isolate #1, Lane 2, isolate #2, Lane 3 Bio-Rad Lambda ladder PFGE marker (Bio-Rad, California, CA, USA). 1B Autoradiography of 1A. (2) Autoradiography of PFGE of seven S1 digested *K. pneumoniae* strains isolated in Benghazi, Libya.

The results obtained showed that *tni*C-like transposons such as Tn*5090* were found to be common in *K. pneumoniae* strains isolated in geographically distant countries, in this particular case Portugal and Libya. Their common occurrence in *K. pneumoniae* suggests that this organism may provide a suitable background for the movement of the non-mobile, yet more common type of class 1 integron, by providing transposition functions *in trans*. Class 1 integron is most often found housed in a damaged transposon that has lost several mobility genes and is often called Tn*5090*-like or Tn*402*-like. Tn*5090* itself is a transposon originally found in *Klebsiella aerogenes* that harbours a class 1 integron, has a complete set of transposition genes (*tniA, tniB, tni*C, *orf6* and *tni*Q) and is functional. Therefore, it can be suggested that class 1 integrons can be divided in two groups: group 1 where class 1 integrons are housed on an active transposon with resistance gene cassettes found adjacent to the *tni*C gene of Tn*5090* (*tni*C-like integron/transposon); group 2 where class 1 integrons are found on an inactive

transposon that is missing the genes *tni*C, *tni*Q and a section of *tni*B. Group 2 class 1 integrons also have the 3' conserved sequence (3'CS) consisting of a fused *qac* and *sul*1 resistance gene cassettes that is absent from group 1. Until recently, nearly all class 1 integron structures found in the sequence data bases and in clinical bacteria were described as belonging to group 2. However, group 1 class 1 integrons are now emerging rapidly in many places worldwide often carrying powerful metallo-β-lactamase gene cassettes (Toleman *et al.* 2007b). Since this form of the class 1 integron has greater mobility it can be expected to disseminate among Gram-negative bacteria quickly. As a consequence, bacteria have at their disposal one more option to disseminate structures that carry genes conferring antibiotic resistance, thus risking the success of antibiotic treatment.

4.2.4 Screening of *qnr* genes

Screening for the *qnr* genes was performed by PCR using the amplification conditions and primers described Material and Methods section 2.3. Fifteen out of the twenty-one isolates tested were positive for the presence of *qnr* genes (Table 4). Twelve of them carried the *qnr*A1 which, in eight isolates, was found downstream of the In*37* and IS*CR1,* which encodes a recombinase and provide the promoter sequences for high-level expression of the plasmid–mediated *qnrA* gene in Enterobacteriaceae. All the isolates co-harbouring *qnrA*, *aac(6')-Ib-cr* and $bla_{\text{OXA-30}}$ also harbored IS*CR1* and a class 1 integron. The *ampR* gene was located downstream of the *qnrA1* gene, in the position where it has been identified in In*36* and In*37*. Three isolates carried the *qnr*B alone and four isolates were positive to both *qnr*A and *qnr*B simultaneously (Figure 25). *qnr*S could not be detected.

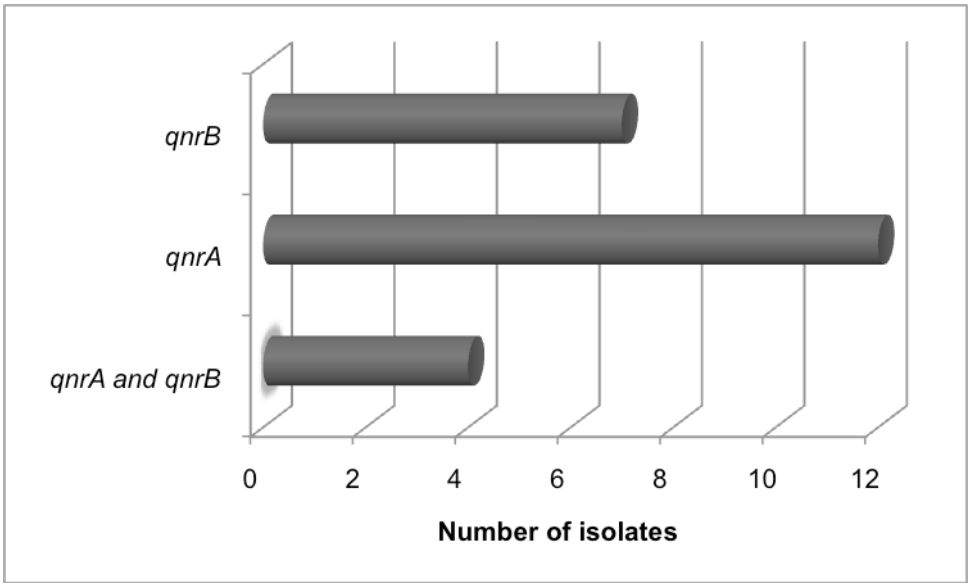


Figure 25 – Distribution of *qnr* among the *K. pneumoniae* isolates being studied.

Previous reports described the expression of *qnrA* and *qnrS* in a *E. coli* clinical isolate from Scandinavia (Cavaco *et al.* 2007a). It has also been reported the co-existance of *qnrB4* and *qnrS* in two different plasmids found in a same clinical *K. pneumoniae* isolate from China (Aihua *et al.* 2008). However, we reported four isolates positive to both *qnr*A1 and *qnr*B4. Also, most of the *qnr* positive isolates were broad-spectrum β-lactamases producers, re-inforcing, once again, a possible relationship between *qnr* genes and broad-spectrum β-lactamases.

These findings indicate an extremely high prevalence of the *qnr* genes associated with various resistance determinants such as IS*CR1* and class 1 integrons. Moreover, the role of the co-existence of both *qnr*A and *qnr*B in the isolates is yet to be clarified, although it can be suggested that it act as a reservoir for further dissemination of these genes to sensitive bacteria.

Table 4 – Characteristics of *qnr*A, *qnr*B and *qnr*A/*qnr*B positive *K. pneumoniae* isolates.

Isolate	MIC μg/mL			*intI1*	IS*CR*	*bla*$_{OXA}$	*bla*$_{CTX-M}$	*bla*$_{TEM}$	*bla*$_{SHV}$	*bla*$_{DHA}$	*qnr*
	NA	CIP	NOR								
KP2	≥32	≥4	8	-	-	-	-	*bla*$_{TEM-1}$	*bla*$_{SHV-1}$	-	*qnrA1*
KP4	≥32	≥4	8	+	-	-	-	-	-	*bla*$_{DHA-1}$	*qnrA1*, *qnrB4*
KP5	≥32	≥4	≥16	+(In*37*)	+	*bla*$_{OXA-30}$	-	*bla*$_{TEM-1}$	*bla*$_{SHV-1}$	*bla*$_{DHA-1}$	*qnrA1*, *qnrB4*
KP6	≥32	≥4	8	+(In*37*)	+	*bla*$_{OXA-30}$	-	*bla*$_{TEM-1}$	*bla*$_{SHV-1}$	-	*qnrA1*, *qnrB4*
KP7	≥32	≥4	≥16	+(In*37*)	+	*bla*$_{OXA-30}$	-	*bla*$_{TEM-1}$	*bla*$_{SHV-5}$	-	*qnrA1*
KP8	≥32	≥4	8	+	+	-	-	*bla*$_{TEM-1}$	*bla*$_{SHV-1}$	*bla*$_{DHA-1}$	*qnrA1*
KP9	≥32	≥4	8	-	-	-	-	*bla*$_{TEM-1}$	-	-	*qnrB4*
KP11	≥32	≥4	8	+	+	-	-	-	*bla*$_{SHV-1}$	-	*qnrA1*
KP10	≥32	≥4	8	+(In*37*)	+	*bla*$_{OXA-30}$	-	*bla*$_{TEM-1}$	*bla*$_{SHV-1}$	-	*qnrA1*, *qnrB4*
KP14	≥32	≥4	8	+(In*37*)	+	*bla*$_{OXA-30}$	-	-	*bla*$_{SHV-1}$	*bla*$_{DHA-1}$	*qnrA1*
KP16	≥32	≥4	8	+(In*37*)	+	*bla*$_{OXA-30}$	-	-	*bla*$_{SHV-1}$	*bla*$_{DHA-1}$	*qnrA1*
KP17	≥32	≥4	8	+	-	-	-	-	*bla*$_{SHV-5}$	-	*qnrB4*
KP19	≥32	≥4	8	+(In*37*)	+	*bla*$_{OXA-30}$	-	-	*bla*$_{SHV-1}$	*bla*$_{DHA-1}$	*qnrA1*
KP20	≥32	≥4	8	+(In*37*)	+	*bla*$_{OXA-30}$	-	*bla*$_{TEM-1}$	*bla*$_{SHV-1}$	-	*qnrA1*
KP21	≥32	≥4	8	+	-	-	-	-	*bla*$_{SHV-1}$	*bla*$_{DHA-1}$	*qnrB4*

NA-Nalidixic Acid; CIP- Ciprofoxacin; NOR- Norfloxacin; (-) negative for the presence of the screened gene; (+) positive for the presence of the screened gene;

4.2.5 Pulse field gel electrophoresis and determination of gene location

PFGE and *S1* digestion were performed as described in Material and Methods section 2.13. PFGE of *S1*-digested genomic DNA revealed the presence of high molecular weight plasmids, ranging from 75 kb to 450 kb, in all strains carrying *qnr* genes. S1-restricted plasmids were hybridised to labeled *qnrA* and *qnrB*-specific probes, as described in Material and Methods sections 2.7.1 and 2.7.2. Results revealed that both genes are plasmid encoded and that *qnr*A1 and *qnr*B4 are located on the same plasmid in two of the strains (KP4 and KP10) and on different plasmids in two strains (KP5 and KP6). *qnr*A1 gene was found on a 450kb plasmid in the strains KP4, KP5, KP6, KP10. *qnr*B4 was found located on the same plasmid (450kb) in two of the strains (KP4 and KP10) and in a 75 kb plasmid in strains KP5 and KP6. This is the first report of the presence of both *qnr*A1 and *qnr*B4 in the same plasmid.

4.2.6 Matting assays

Conjugation was attempted using *E. coli* J53 Azi^r as the recipient strain according the methodology described in Material and Methods section 2.6.2. Neither of the *qnr* genes could be transferred to *E. coli* J53 Azi^r, under the laboratory experimental conditions. Suggestions can be given to justify these results: 1) the plasmids carrying *qnr*A and *qnr*B are of such a large size (450kb) thus carrying an enormous amount of foreigner DNA, which can lead the cell machinery to degrade it; 2) Also, the plasmid may be broken during conjugation and may not be able to re-circularize, which once again leads to DNA degradation. Despite the fact the conjugation experiment failed under laboratory conditions, since the *qnr* genes are located on a plasmid it can promote its dissemination. In its natural environment, exchange of DNA between bacteria can occur when appropriate conditions in the surroundings are achieved. Under laboratory limitations, those conditions are not always easy to gather or adjust.

4.2.7 General Conclusion

All the *K. pneumoniae* isolates included in this study were MDR. Sputum was the biological product from which a higher number of isolates was retrieved. IS*CR1* was detected in eight isolates closely associated with In*37*. bla_{oxa-30} when detected was also associated with the class 1 integron In*37*. The bla_{CTX-M} gene was not detected in any isolate. *int1* was detected in 81% of the isolates and the array most frequently found was [*dfrA12-aadA2*]. Surprisingly 17 isolates carried an integron inserted in a *Tn*5090 transposon. Fifteen isolates carried quinolone resistance genes. Among these, four harboured the quinolone resistance genes *qnr*A and *qnr*B, both located on a large plasmid in two isolates (KP4 and KP10). In two of the isolates (KP5 and KP6) those genes were found on different plasmids. Overall, these findings indicate an extremely high prevalence of the *qnr* genes associated with various resistance determinants such as IS*CR1* and class 1 integrons.

5. Chapter 3 – Carriage of *qnr*A1 and *qnr*B2, *bla*$_{\text{CTX-M-15}}$ and complex class 1 integron in a clinical multi-resistant *Citrobacter freundii*

5.1 Introduction

Among *Citrobacter* species, the most commonly isolated from human clinical specimens are *Citrobacter koserii, Citrobacter freundii, Citrobacter youngae, Citrobacer braakii, and Citrobacter amalonaticus*. Nevertheless, the majority of infection cases are associated with *Citrobacter koseri* and *C. freundii* (Samonis *et al.* 2009). The latter is an opportunistic pathogen that can cause diarrhoea, septicemia, meningitis, urinary tract and respiratory system infection, especially in high-risk groups (Zhang *et al.* 2009a). *C. freundii* is member of the Enterobacteriaceae and in China it has been ranked tenth among Gram-negative organisms considered nosocomial resistant pathogens, despite being traditionally considered to have low level of virulence (Zhang *et al.* 2009a). A study performed by Samonis and co-workers (2009) revealed that the isolation of *Citrobacter* species, although rather infrequent, was clinically relevant in the vast majority of cases. Therefore, as suggested by Samonis and co-workers (2009), further attention should be given to these pathogens.

Quinolones are broad-spectrum antibiotics that have been widely used in clinical practice (Andriole 2005). The increasing use of fluoroquinolones has triggered an increase of resistance in Enterobacteriaceae that originally were highly susceptible to this class of antibiotics (Robicsek *et al.* 2006). *qnr* genes have been hypothesized to contribute to the increasing prevalence of quinolone resistance among Gram-negative bacteria. Epidemiological surveys around the world have found *qnrA*, *qnrB*, and *qnrS* in various Enterobacteriaceae such as *C. freundii, E. coli, Enterobacter cloacae, Enterobacter sakazakii* and *K. pneumoniae* (Cavaco *et al.* 2007b). Frequently, quinolone resistance and ESBL production are associated. This can be due to the plasmid mediated ESBL and *qnr* genes that are often associated with mobile elements, such as integrons and IS*CR* elements (Machado *et al.* 2005). This study is focused on a *C. freundii* possessing a wide range of antibiotic resistance determinants, such as integrons, *bla*$_{CTX-M-15}$, IS*CR1* and *qnr* genes.

5.2 Results and Discussion

5.2.1 Clinical context of CIT1 recovery

The *C. freundii* isolate, CIT1, was recovered from a 17-year-old boy admitted in mid-August 2008, with the diagnosis of dorso-lumbar spondylodiscitis caused by methicillin-sensitive *Staphylococcus aureus* (MSSA). The patient was initially treated with flucloxacillin (2g q4h IV) and subsequently with teicoplanin (12mg/Kg od IV, with initial loading dose). The patient was henceforth discharged, and teicoplanin treatment continued to be administered as an outpatient. After discharge, clinical signs (severe chills, cyanosis of lips and extremities and, afterwards, fever) of a bloodstream infection were observed. The patient was re-admitted, maintaining the teicoplanin treatment, and imipenem 1gq 8h IV was empirically started. Within the central venous catheter grew a Gram-negative bacillus (CIT1 specimen), later identified as *C. freundii*. Imipenem treatment was maintained for 14 days, and the patient recovered.

5.2.2 Species identification and antibiotic susceptibility testing

Species identification was performed using the VITEK2 Compact system and the AES (bioMérieux, Marcy L´Étoile, France). AES highlighted six MDR *C. freundii,* during 2008. Among these isolates one particular isolate, CIT1, isolated from blood, showed a high antibiotic resistance profile. Susceptibility testing was carried out according to guidelines of CLSI standards (CLSI 2007).

C. freundii, CIT1, was resistant to all β-lactams tested: ampicillin (≥ 32 μg/mL), ampicillin/ sulbactam (≥ 32 μg/mL), amoxicillin/ clavulanic acid (≥ 32 μg/mL), cefotaxime (≥ 64 μg/mL), ceftazidime (≥ 32 μg/mL), cefepime (≥ 32 μg/mL), piperacillin (≥ 128 μg/mL) and piperacillin/tazobactam (≥ 128 μg/mL). Susceptibility was only shown to the carbapenems: imipenem (≤ 1 μg/mL) and meropenem (≤ 1 μg/mL). Resistance to aminoglycosides: gentamicin (≥ 16 μg/mL) and tobramycin

(≥ 16 μg/mL); and to fluoroquinolones: ciprofloxacin (≥ 4 μg/mL) and norfloxacin (≥ 16 μg/mL), was also observed. ESBL production was detected using Etest ESBL with cefotaxime/ cefotaxime + clavulanic acid (CT/CTL), ceftazidime/ ceftazidime + clavulanic acid (TZ/TZL) and cefepime/ cefepime + clavulanic acid (PM/PML) strips according to the manufacturer's instructions (AB bioMérieux, Solna, Sweden). Etest CT/CTL and TZ/TZL gave an inconclusive result, but PM/PML confirmed that CIT1 was an ESBL producer (Figure 26). The inconclusive results obtained with CT/CTL and TZ/TZL Etest strips may be explained by the presence of an ampC β-lactamase. It is known that *C. freundii* possesses a chromosomal CMY AmpC and it is also known that clavulanic acid induces its expression, thus enhancing the resistance.

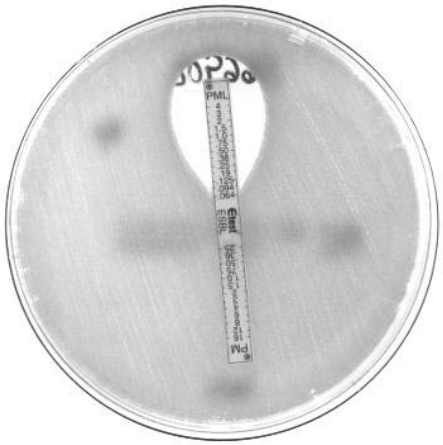


Figure 26 – Muller-Hinton agar (bioMérieux, France) plate showing a PM/PML Etest strip (bioMérieux, France) positive result for the presence of an ESBL in the *C. freundii* isolate.

5.2.3 Matting assays

Conjugation experiments were performed using *E. coli* J53 Azi^r as the recipient strain as previously described in Material and Methods section 2.6.2. Conjugation was unsuccessful, under the various laboratory experimental conditions tested. *qnr*A and *qnr*B were detected in the plasmid DNA but not in the chromosomal DNA of *C. freundii* CIT1, implying that *qnr*A or *qnr*B were plasmid encoded. Hybridization experiments confirmed that *qnr*A and *qnr*B are in located on the same plasmid of 450kb. The large size of the plasmid can explain the unsuccessful conjugation assay.

5.2.4 IS*CR,* class 1 integrons and β-lactamase genes content

PCR was performed to detect the presence of β-lactamase genes belonging to the *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{OXA}$ groups, plasmidic AmpC (DHA, MOX, FOX, CMY and ACC) families, as well as *qnr* genes, class 1 integrons*,* IS*Ecp1*, IS*CRs* and *tniC* transposons. Amplification conditions and primers used were previously described in the Material and Methods section 2.3. Nucleotide sequence of PCR products was determined and compared to others deposited in the European Molecular Biology Laboratoty (EMBL) Genebank. PCR and nucleotide sequence analysis revealed the presence of IS*Ecp1* linked to a *bla*$_{CTX-M-15}$ (Accession number FJ459818), which may explain the resistance exhibited by this isolate to third generation cephalosporins, as previously described by Eckert *et al*, 2006. IS*Ecp1* has been reported to act as a strong promoter positively regulating the expression of *bla*$_{CTX-M-15}$ and also responsible for its mobilization (Poirel *et al.* 2003). Genes encoding *bla*$_{OXA-30}$ and *bla*$_{TEM-1}$ were also detected and may contribute for the penicillin resistance exhibited by CIT1, namely to ampicillin. As expected, the CMY *amp*C was also detected in CIT1 once that it has been accepted that CMY originates from the chromosome of the *C. freundii* species (Barlow and Hall 2002; Hanson 2003).

In typical *E. coli* and *Shigella* species, *ampC* is expressed at such low levels that deletion of *ampC* does not affect sensitivity to β-lactams. However, in *C. freundii* and *Enterobacter cloacae*, *ampC* is inducible by both penicillins, such as ampicillin, and by cephalosporins (Barlow and Hall 2002). Due to their location on the host chromosomes, *ampC* genes were not initially subjected to the rapid dissemination promoted by the horizontal transfer of plasmid-borne genes (Barlow and Hall 2002). However, some *ampC* genes jumped into plasmids, which enhanced their fast dissemination between species and around the world.

This is also the first description of a complex class 1 integron structure [*dfrA12, orfF, aadA2*]:IS*CR1:qnrA1* in *C. freundii* (Figure 27). Interestingly, class 1 integron array [*dfrA12, orfF, aadA2*] has been reported associated with the *sul3* gene in *E. coli* strains from animal sources in Norway and Denmark (Sunde *et al.* 2008). This suggests a dissemination of this structure, in different genetic contexts and environments, but in either case plasmid located.

Figure 27 – Schematic representation of the class 1 integron associated with IS*CR1* and *qnrA1* that was found in the CIT1 isolate. Light grey box- antibiotic resistance genes cassettes; black box- integrase gene, silver box, IS*CR1*; white box, *qnrA1*. Arrows indicate the direction of transcription. Solid black circles represent 59-base elements and grey ellipses represent the *att*I1 site of the integrants found in the *C. freundii* isolate CIT1.

5.2.6 Screening of *qnr* genes

PCR amplification and nucleotide sequence analysis of the resulting amplicon, revealed the presence of both *qnr*A1 and *qnr*B2 in CIT1 plasmid DNA (Figure 28). Their genetic context and location was further investigated. Amplification conditions and primers were previously described in Material and Methods section 2.3. Plasmid DNA was extracted by alkaline lyses according to the protocol described in Material and Methods section 2.5.2.

Hitherto, *qnr*A1*,* has never been found in *C. freundii*, and in CIT1 it is associated with a class 1 integron, [*dfrA12, orfF, aadA2*] and IS*CR1* as shown in Figure 28*. qnrA1* was found downstream of the *ISCR1*. The class 1 integron has been previously described within a highly transmissible plasmid in a *C. freundii* isolate (Acession Number NC_004464). However, in that particular case it was neither associated with IS*CR1* nor with a *qnrA* gene. Despite being carried on the same plasmid, *qnr*B2 (Accession Number FJ232918), was not found associated with either IS*CR1*, *tni*C or IS*Ecp1*. Its genetic context could not be determined, despite all the attempts made and based on the genetic contexts previously described in the literature, for other *qnr*B genes.

This is the first report of the presence of *qnr*A1 and *qnr*B2 in *C. freundii* isolate. Quinolone MIC value does not reflect a clear advantage to this strain of carrying both genes, when compared to other bacteria carrying only one of the genes. However, it might contribute to an increased resistance of the strain when subjected to quinolone selective pressure. Although responsible for low-level quinolone resistance, in the presence of other encoded resistance genes/mechanisms, *qnr* genes may be an important tool for bacteria to avoid lethal quinolone concentrations.

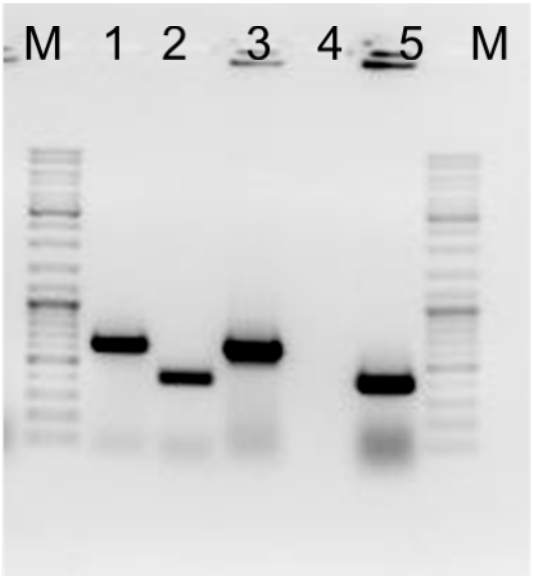


Figure 28 – PCR amplification of *qnr*A and *qnr*B in CIT1 plasmid DNA; M – DNA size marker GeneRuler Ladder mix (MBI Fermentas, Vilnius, Lithuania); 1 – *qnr*A gene; 2 – *qnr*B gene; 3 – *qnr*A positive control; 4 – DNA negative control; 5 – *qnr*B positive control.

5.2.7 Pulse field gel electrophoresis and determination of gene location

PFGE and *S1* digestion of genomic DNA were performed as described in Material and Methods section 2.7.11. Hybridisation and DNA probe labeling were performed according to the methodology described in sections 2.7.1 and 2.7.2.

PFGE of *S1*-digested genomic DNA revealed the presence of two plasmids in CIT1 isolate. *C. freundii* isolates carrying more than one plasmid have already been described by other authors (Sekiguchi *et al.* 2008; Zhang *et al.* 2009a). Previous studies reported that plasmids with higher molecular weight were associated with several antibiotic resistance genes, such as those conferring resistance to β-lactams (Sekiguchi *et al.* 2008). Nevertheless plasmids with the molecular weight ranging between 40-450Kb such as those found in CIT1 strain have never been described in *C. freundii.*

Hybridisation results revealed that *qnr*A and *qnr*B genes were located on the same large plasmid of approximately 450kb as shown in figure 29.

Moreover, *qnr*A1 and *qnr*B2 (Accession number FJ232918) were located on the same plasmid and are here reported for the first time in this species and with this location; nevertheless other studies performed with *K. pneumoniae* clinical isolates, reported the presence of these two genes also on the same plasmid (Ferreira *et al.* 2008). Interestingly, both isolates (*K. pneumoniae* and *C. freundii*) carrying both *qnr*A1 and *qnr*B2 genes in large plasmids (450kb), were recovered from different biological specimens (sputum and catheter, respectively) from inpatients in distinct wards (Intensive Care Unit and Infectious Diseases, respectively) within the same hospital and in different timeframes. However, in both cases, *qnr*A1 was associated with a class 1 integron and an IS*CR1*, and this complex structure was located on a plasmid of similar size. This fact suggests the potential of dissemination of these resistance determinants. In fact, hybridisation data suggest that all these structures are found on the same plasmid permitting their further dissemination.

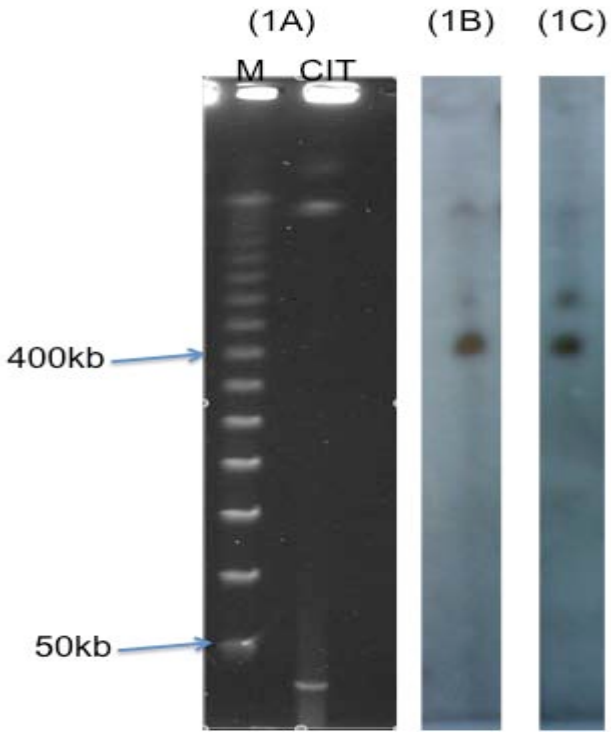


Figure 29 – (1A) M – Bio-Rad lambda ladder PFGE marker (Bio-Rad, California, CA, USA); CIT - *C. freundii* DNA digested with S1 nuclease. Arrows denote molecular weights of the marker; (1B) Autoradiography of (1A) with *qnrA* probe: (1C) Autoradiography of (1A) with *qnrB* probe.

5.2.8 IS*CR1* as *qnrA1* promoter

It has been reported that IS*CR1,* encoding a recombinase, provides the promoter sequences necessary for high-level expression of plasmid–mediated *qnrA* gene in Enterobacteriaceae (Rodriguez-Martinez *et al.* 2006). Figure 30 shows the multiple alignments of *C. freundii* (CIT1)*, E. coli* (GenBank accession AM295981.1) and *K. pneumoniae* (GenBank accession EF682136) IS*CR* nucleotide sequences, including 33 bp DNA sequence containing *ori*IS and the start codon of the *qnr*A gene. ClustalW comparison of these sequences, revealed a strong homology with the *C. freundii* promotor sequence. It also shows that IS*CR1*, as revealed by the comparison with other sequences, promotes *qnr*A1 expression in CIT1. Furthermore, analysis of the genetic environment surrounding the 3' end of the DNA sequence containing *ori*IS revealed that the upstream sequence of *qnr*A in CIT1 and *E. coli* had an additional 69 bp compared to the upstream sequence of

K. pneumoniae. This indicates that the variation in these genetic environments might be due to past transposition events by the IS*CR1* upstream of the *qnr*A gene, as also reported by other authors (Sohn *et al.* 2009).

Figure 30 – Comparison of the *C. freundii* IS*CR1* nucleotide sequence (GenBank accession FJ266018) with other IS*CR1* nucleotide sequences (GenBank accession AM295981.1 and EF682136). IS*CR1* Ori*IS* of transposition is represented by the black rectangle and initiation codon of *qnr*A gene is represented by the dashed rectangle.

5.2.9 General Conclusion

The MDR *C. freundii* recovered from a catheter of a patient, carried an IS*CR1*, a *qnr*A1 and *qnr*B2, *bla*$_{CTX-M-15}$ and a complex class 1 integron that is described for the first time in this species. This isolate harboured the quinolone resistance genes, *qnr*A1 and *qnr*B2, both located on a large plasmid (450kb). Analysis of the

nucleotide sequence of IS*CR1* showed that it provides a promoter for the *qnr*A gene associated with it. Moreover, the results also suggest that isolates, which are not commonly linked to sepsis, rather to opportunistic infections, can be a reservoir of antibiotic resistance genes that can be transferred to other bacteria. Therefore these reservoirs represent a threat within the hospital environment.

6. Chapter 4 – Association of IS*CR2* with antibiotic resistance genes in Portuguese *Acinetobacter baumannii* isolates

6.1. Introduction

Acinetobacter spp., specially *Acinetobacter baumannii,* are frequently associated with nosocomial infections, particularly in intensive care units, where their ability to resist to dryness can pose substantial problems in their eradication from the environment. They have also been recognized as invaders of burn wounds (Hawkey 2008; Livermore and Woodford 2006). *Acinetobacter* spp. produce a wide range of β-lactamases and have a formidable spectrum of intrinsic resistance mechanisms, meaning that some strains are resistant to all known antibiotics with the exception of colistin (Hawkey 2008). It is also notoriously associated with outbreaks, facilitated by resistance to disinfectants and desiccation (Livermore and Woodford 2006). Some strains of *A. baumannii* became MDR by sequestering antibiotic resistant genes present on plasmids, transposons, or integrons carrying arrays of genes encoding resistance to several antibiotic families (Fournier *et al.* 2006). IS*CR* elements are becoming increasingly important due to their association with various antibiotic resistance genes. IS*CRs* can be broadly classified into 2 groups, IS*CR* associated to class 1 integrons and IS*CR* non associated with class 1 integrons (Toleman and Walsh 2008). The recent genomic sequence of the *A. baumannii* strain AYE by Fournier *et al.* (2006) revealed the presence of two IS*CR* elements, IS*CR1* as part of a complex class integron and IS*CR3*; however, hitherto these are the only two IS*CR* elements to be denoted from this organism (Poirel, personal communication).

IS*CR2* element sequences deposited in DNA data-bases are invariantly found adjacent to antimicrobial resistance genes although they are usually described as "putative transposases" and not IS*CR* elements. IS*CR2* is intimately associated with antimicrobial resistance genes especially sulphonamide (*sul*2) and trimethoprim (*dhfr*9, *dhfr*18, *dhfr*20) in *Escherichia coli*, *Stenotrophomonas maltophilia* and *Vibrio cholerae* (Toleman *et al.* 2006b).

IS*CR* database: http://www.cardiff.ac.uk/medic/aboutus/departments/medicalmicrobiology/genetics/iscr.html

IS*CR2* has also been found located on a number of plasmids adjacent to chloramphenicol/florphenicol resistance gene, *flo*R, sometimes on the novel integrative and conjugative multidrug resistance encoding genetic element SXT. IS*CR2* elements have now been found in several bacterial species and in different geographical and ecological environments (Toleman *et al.* 2006b).
This study describes the first cases of IS*CR2* in *A. baumannii* from Portugal as well as their novel genetic context. Retrospective studies also show the presence of IS*CR2* since 1995.

6.2 Results and Discussion

6.2.1 Background of the *A. baumannii* isolates

Seventeen MDR *A. baumannii* were recovered from various clinical specimens (urine (5), pus (4), sputum (7), and blood (1) Figure 31) that were collected in the Hospital Infante D. Pedro EPE, Aveiro, central region of Portugal, in different timeframes (2006, 2007 and 2008). All of the isolates were recovered after 72 h of patient admission, thus suggesting a nosocomial infection. Acb17 isolate was collected in 2006, it was a MDR isolate causing respiratory tract infection and ultimately the patient's death. This particular isolate was further studied, since it was recovered from a patient hospitalized in the ICU new facility. At that time this episode caused extreme concern since the source of contamination was not identified and the possibility of an outbreak was considered, as it later turned out to be true. The isolates collected in 2007 were recovered during an outbreak that occurred in the hospital and spread to the different wards. In 2008, after the implementation of several decontamination measures, two isolates (Acb15 and Acb16) were again collected from patients hospitalised in the ICU.
A. baumannii isolates collected in Coimbra (one isolate) and in Argentina (two isolates) were included in this study merely for comparison purposes. The isolate collected in Coimbra, AcbHUC, was used for resistance profile comparison and

also as positive control to the presence of *bla*$_{OXA-40}$. The Argentinean isolates were used as positive controls for the presence of IS*CR2* and for genetic context comparison.

A higher number of isolates were recovered from sputum, as expected, since most of these patients have been diagnosed with a respiratory tract infection. It is, however noteworthy that from other specimens, with the exception of blood, similar number of isolates were recovered in the different specimens, suggesting the ubiquity of this species and its adaptability to different conditions, and ultimately to cause infection.

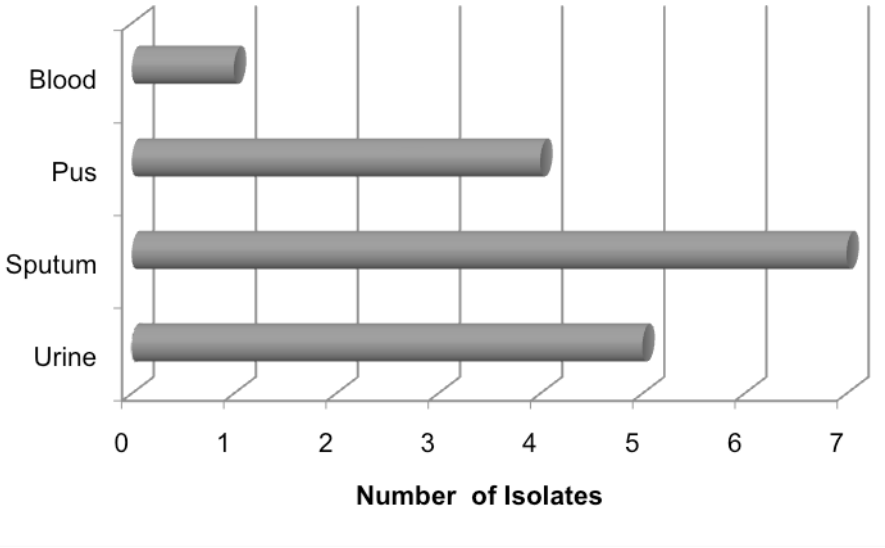


Figure 31 – Biological products from which the *A. baumannii* isolates were recovered are indicated in the vertical axis. The number of isolates recovered from each product is indicated in the horizontal axis.

The patients age was comprised between 45 and 87 years old. The majority of the isolates were recovered from patients with ages above the 60 years. The *Acinetobacter* species are commonly found associated to nosocomial infections that frequently become colonizations. Thus it is predictable that patients with extended hospitalization periods are likely to be infected, independent of their age. It is common sense that the elderly that often need medical care and are more debilitated and thus more exposed to *A. baumannii* nosocomial infections.

However, and among the population studied, some isolates were retrieved from middle age patients, which suggest that in this case and during an outbreak the probability of an infection caused by an *A. baumannii* is age independent.

6.2.2 Species Identification and antibiotic susceptibility testing

Species identification was performed using in the VITEK2 system and AES (bioMérieux, Marcy L´Étoile, France). Susceptibility testing was carried out according to the guidelines of CLSI standards (CLSI 2007).

Antibiotic susceptibility testing revealed, as expected, that the *A. baumannii* isolates collected were MDR, exhibiting a similar multidrug resistance pattern. As described in other studies performed in Portugal, MDR *A. baumannii* has increasingly been recognized as responsible for large and sustained outbreaks of nosocomial infections (Silva *et al.* 2007). The *A. baumannii* population studied showed a resistance profile similar to that described by da Silva and colleagues (2007) in isolates collected from different hospitals in the country. The isolates included in the present study were resistant to all the β-lactams tested including resistance to amoxicillin and to the association of amoxicillin with clavulanic acid, ureidopenicillins and their associations, cefoxitin, ceftriaxone, ceftazidime, cefepime, cefpirome, aztreonam (MICs between 64 and > 256 μg/mL), ampicillin/sulbactam (MIC of 64 μg/mL) and resistance to imipenem (MIC >16 μg/mL). Fifteen isolates were resistant (MIC ≥ 4 μg/mL) and two were susceptible to ciprofloxacin (MIC < 4 μg/mL). The susceptibility profile to aminoglycosides was variable. Seven isolates were susceptible to gentamicin whereas eight were susceptible to tobramycin. MICs of representative antibiotics of each class are listed in Table 5. All the isolates were susceptible to colistin. Thus, colistin and in some cases aminoglycosides were the antibiotics left in the narrow list of options to treat infections caused by these *A. baumannii* isolates.

Table 5 – Demographic details, MIC values and presence of IS*CR2* and class 1 integrons in *A. baumannii* population included in this study.

Strains	Collection year	MIC (μg/mL)						IS*CR2* Positive	Class 1 integron
		IMI	CTZ	ATZ	GENT	TOB	CIP		
Acb1	2007	≥16	≥64	>64	1	1	≥4	+	+
Acb2	2007	≥16	≥64	>64	2	1	≥4	+	+
Acb3	2007	≥16	≥64	>64	1	1	0.5	-	-
Acb4	2007	≥16	≥64	>64	≥16	0.5	≥4	+	+
Acb5	2007	≥16	≥64	>64	2	1	≥4	+	+
Acb6	2007	≥16	≥64	>64	1	1	≥4	+	+
Acb7	2007	≥16	≥64	>64	≥16	8	≥4	+	+
Acb8	2007	≥16	≥64	>64	≥16	8	≥4	+	+
Acb9	2007	≥16	≥64	>64	≥16	>16	≥4	+	+
Acb10	2007	≥16	≥64	>64	1	1	0.5	-	-
Acb11	2007	≥16	≥64	>64	≥16	8	≥4	+	+
Acb12	2007	≥16	≥64	>64	1	1	≥4	+	+
Acb13	2007	≥16	≥64	>64	8	8	≥4	+	+
Acb14	2007	≥16	≥64	>64	8	8	≥4	+	-
Acb15	2008	≥16	≥64	>64	8	8	≥4	+	+
Acb16	2008	≥16	≥64	>64	8	8	≥4	+	+
Acb17	2006	≥16	≥64	>64	8	8	≥4	+	+
AcbHuc	1995	≥16	≥64	>64	8	8	≥4	+	-

IMI- imipenem; CTZ- ceftazidime; ATZ- aztreonam; GENT- gentamicin; TOB- tobramycin; CIP- ciprofloxacin

6.2.3 Characterization of IS*CR2* genetic context

Amplification conditions and primers used are described in Material and Methods section 2.3. Fifteen out of the seventeen isolates were positive for the presence of the IS*CR2* element. The *A. baumannii* isolates tested seem to be identical in that they possessed only a single copy of the IS*CR2*. The genetic context of the further to the left and right hand side of the IS*CR2* was investigated using the methodology described in Material and Methods section 2.8. Results obtained revealed a truncated *lys*R gene followed by a resolvase gene to the left hand side of IS*CR2* and a truncated phospho-glucosamine mutase gene, *glm*M immediately to the right hand side followed by a *sul*2 gene (Figure 32). Analysis of this locus indicated that the G+C content of each reading frame was 59.9-60.3% and does not derive from the *Acinetobacter* spp. (average G+C content of 39%), but may have collectively originated from a single organism with a G+C % of approx. 60%. Comparisons with other IS*CR2* structures in the database showed that these sequences are unique in that they possess the *sul2* gene. The only other structure reported thus far not to have a second part-copy of IS*CR2* was reported from *Stenotrophomonas maltophilia* isolated from the USA in 2006 (accession number AM181666).

Sequence analysis of the 7150bp IS*CR2* locus from the two *A. baumannii* collected in Argentina and used for comparison (Acb23 and Acb25) indicated that two identical IS*CR2* elements (referred to as IS*CR2A* and IS*CR2B*) were found flanking a 2.4 kb section of DNA. This section included a *flo*R resistance gene and a 305bp transcriptional regulator gene encoding a LysR-type protein immediately downstream of *flo*R; the second copy of IS*CR2* was also adjacent to a *sul*2 gene and a truncated *glm*M (Figure 32). Comparison of these two tandem repeated IS*CR2* elements showed that they are almost identical. Alignment of the IS*CR2A/B ori*IS sequences (to the right hand side of IS*CR2*) and adjacent DNA revealed identical sequence until the junction between the *ori*IS and the adjacent sequence, where the identity was abruptly lost. This indicates that the two IS*CR2* elements were originally produced by replicative transposition events rather than a duplication event through homologous recombination.

Comparisons of the genetic context of the IS*CR2*/*flo*R/IS*CR2* fragments from Acb23 and Acb25 to similar sequences in the databases revealed a number of structures containing *flo*R gene also flanked by full length or truncated copies of IS*CR2* genes. The sequence of this section has high identity and also the same gene arrangement found in the *E. coli* BN10660 plasmid (Accession number AF231986) isolated in France in 2000. A similar IS*CR2* region has been reported where the authors detected circular intermediate forms of the element and related this to the potential mobility of *flo*R gene (Doublet *et al.* 2005). In three other cases, this gene arrangement is similar to sequences from *Vibrio cholerae* strain V21 isolated from Viet Nam in 2003 (AB114188), from plasmid pAb5S9 isolated from *Aeromonas bestiarum* in France in 2007 (EF495198) and from plasmid pSN254 isolated from *Salmonella enterica* SL254 in 2007 (CP000604) where *flo*R gene is flanked by a full copy of the IS*CR2* gene on left hand side, but only has 300-500bp of a truncated IS*CR2* gene on the right hand side. Other *flo*R genes have adjacent and truncated copies of IS*CR2* on the left right hand side as it has been found in isolates from Germany and India in 2005, which have arisen as a result of a deletion event.

This was the first report of the presence of an IS*CR2* in *A. baumannii* and highlights the universal and the unusual antibiotic resistant mobile element distributed in different geographical locations. South American isolates were chosen as has been shown that IS*CR4* is associated with bla_{SPM-1} (Poirel *et al.* 2004) and it would be insightful to see if this had spread to other species. Interestingly both structures possess *sul*2 but only the Argentinean isolates possess *flo*R suggesting that one structure has evolved from the other. The insertion of a second copy of IS*CR2(A)* and *flo*R by a transposition event was followed by homologous recombination.

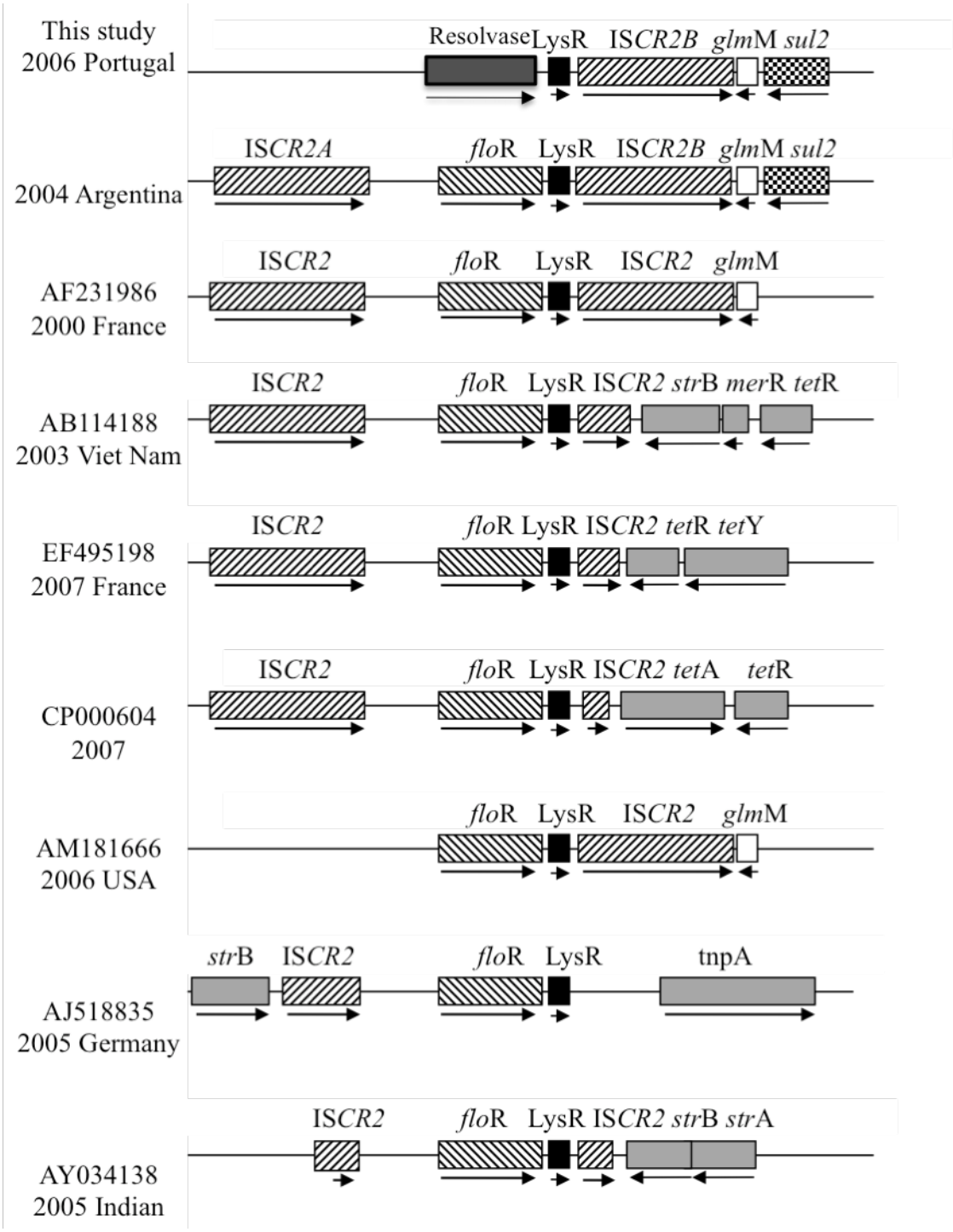


Figure 32 – Genetic context of IS*CR2* fragment compared with similar regions containing IS*CR2*. Open reading frames are indicated with open boxes and the direction of their transcription indicated with arrows below the boxes. The IS*CR2* elements are denoted by diagonal lines reading left to right. *flo*R gene denoted by diagonal lines reading right to left; *lys*R a black box; *glm*M a white box, *sul*2 a chequered box and resolvase gene a dark grey box. All other open reading frames are denoted by grey boxes.

6.2.4 Pulse-field gel electrophoresis and hybridisation experiments

PFGE was performed as described the Material and Methods in section 2.11 using *SmaI* endonuclease (MBI Fermentas, Vilnius, Lithuania). PFGE DNA profiles obtained by of the aforementioned isolates were separated in four different clusters, according to the Tenover criteria (Tenover *et al.* 1995). One particular Portuguese cluster in which the isolates differ by only two or less bands, revealed the presence of a bla_{OXA-40} gene. These clones were already identified and their dissemination has been fully described by Da Silva and colleagues (2004), as belonging to the European clone. Unsurprisingly, the Argentinean strains showed a distinctively different pattern from that of the Portuguese isolates.

S1 digestion of chromosomal DNA clearly revealed the presence of large plasmids in eleven strains (Figure 33). Plasmids size ranged from 180kb (4 isolates) to 250kb (7 isolates). The results from the IS*CR2*-specific hybridisation performed on the S1-digested plasmids demonstrated that the IS*CR2* is not plasmid encoded since no positive hybridisation signal from non-chromosomal DNA fragments was observed further confirming that in all isolates, IS*CR2* is present on the chromosome (Figure 33).

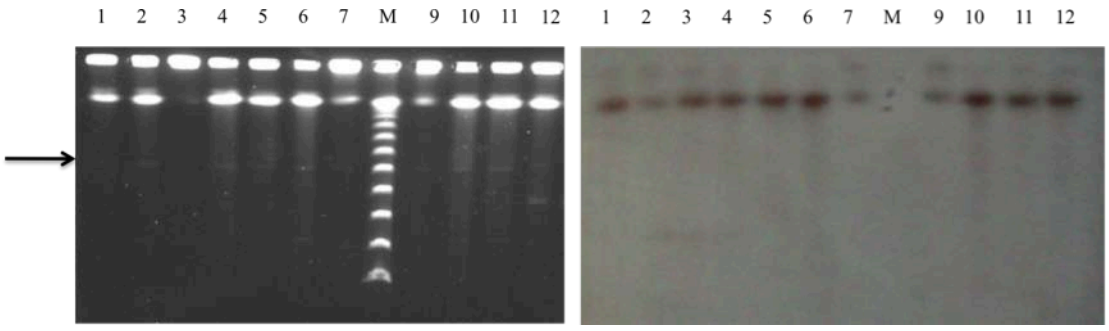


Figure 33 – IS*CR2* hybridisation of S1 digested *A. baumannii* DNA. Lanes 1-7 and 9-12 represent Acb1-Acb7 and Acb9-Acb12, respectively. Lane 8: Bio-Rad lambda ladder PFGE marker (Bio-Rad, Richmond, CA, USA). Arrow indicates the presence of high molecular weight plasmids.

6.2.5 Class 1 integrons and β-lactamase genes content

The presence of class 1 integrons in *A. baumannii* has been significantly associated with multiple antibiotic resistance and the nosocomial dissemination of these isolates has been described worldwide (Luna and Aruj 2007). Amplification conditions and primers used were previously described in the Material and Methods section 2.3. Screening of *intI1* gene in the population under study and the subsequent variable regions amplification in the positive isolates, revealed an expected genetic stability with limited diversity of gene cassettes. PCR amplification and nucleotide sequence analysis of the resulting amplicons, revealed the presence of only two distinct arrays. The gene cassette array found [*aac(3)Ia-orfX-orfY-aadA1*] was described in 2001, in an *A. baumannii* clinical strain from Italy (Accession number AJ310480). The second integron found contained a single gene cassette in its variable region and an *aadA1* gene encoding aminoglycoside resistance. Similar results were reported in a study performed with isolates collected from the major Portuguese teaching hospitals (Pereira *et al.* 2008). Although a high prevalence of genes encoding aminoglycoside-modifying enzymes have been reported among *A. baumannii* integrons, they contribute for a limited resistance. Nevertheless, similar integrons can be found in genotypically distant *Acinetobacter* spp. isolates from distant locations suggesting their dissemination worldwide (Looveren and Goossens 2004).

One of the main concerns about antimicrobial resistance in *A. baumannii* has been the acquisition of carbapenem resistance mainly through the acquisition of class B and D carbapenemases. Screening by PCR for carbapenemase encoding genes revealed the presence of $bla_{\text{OXA-40}}$ in seven isolates (41%). The presence of the promoter sequence, IS*aba1* was also investigated by PCR and was found upstream of the $bla_{\text{OXA-40}}$ in all the positive isolates. As reported by other authors IS*aba1* insertion sequence can act as a strong promoter of $bla_{\text{OXA-40}}$ conducting to its hiper-expression, which leads to carbapenem resistance (Heritier *et al.* 2005). Long-term dissemination of a $bla_{\text{OXA-40}}$ producer *A. baumannii* in the Iberian

Peninsula has been reported by da Silva and co-workers, thus unsurprisingly it was detected in our hospital (Da Silva *et al.* 2004).

6.2.6 Retrospective studies on the *A. baumannii*, AcbHUC

During the course of the study, it was deemed prudent to use an established strain as a negative control for IS*CR2* and *sul*2 screening and for this purpose, AcbHUC was chosen once it was first isolated in 1995. Surprisingly, AcbHUC was shown to possess an identical structure to that found in the other Portuguese isolates derived from 2007. IS*CR2* was retrospectively shown to be part of the RSF1010 plasmid (Radstrom and Swedberg 1988) and therefore has been circulating for decades but so far this is the earliest report of it presence in clinical bacteria.

6.2.7 General Conclusion

The vast majority of the *A. baumannii* population studied was found to be only sensitive to colistin and to the aminoglycosides. IS*CR2* was present in seventeen isolates, is chromosomally located and it is embedded within a novel genetic context. Large plasmids (180 kb and 250 kb) were detected in eleven isolates. Screening of class 1 integrons revealed a genetic stability and only two distinct arrays were found. $bla_{\text{OXA-40}}$ was detected in seven isolates and in all of them it is associated with IS*aba1* representing a major threat since the latter it is known to act as a strong promoter. This association has been reported to be responsible for carbapenem resistance (Heritier *et al.* 2005). The endemic *A. baumannii*, here described was found to belong to the European clone II that is disseminated since 1998 in some Portuguese hospitals. These MDR microorganisms represent a serious problem since a limited choice of antibiotics can be used to treat infections caused by these bacteria.

7. Chapter 5 – Screening of IS*CR* elements in environmental isolates

7.1 Introduction

Nowadays, antibiotics are widely used in human and animal health or as food additives to promote animal growth rate (Kemper 2008). A negative consequence of the present panorama is the fact that the majority of these antibiotics are excreted unchanged into the environment. Thus, concerns about the potential impact of antibiotic residues in the aquatic environment have increased in recent years (Zhang *et al.* 2009b). Moreover, antibiotic resistance in the environment is becoming highly relevant to human health mainly due to the increasing importance of zoonotic diseases as well as the need for predicting emerging resistant pathogens (Allen *et al.*). However, although the scientific community agrees that the use of antibiotics may accelerate the development of antibiotic resistance genes in bacteria, the environmental reservoirs of resistance determinants are poorly understood. The emergence of antibiotic resistance genes in the water environment is becoming an increasing source of concern worldwide. Hundreds of various genes encoding resistance to a broad range of antibiotics have been found in microorganisms distributed in both hospital and animal production wastewaters, but also in sewage, wastewater treatment plants, surface water, groundwater, and even in drinking water (Zhang *et al.* 2009b). The emergence of those genes in aquatic environments is a consequence of the intensive antibiotic usage in hospitals, swine production areas, and fish farms (Chee-Sanford *et al.* 2001). Among the genetic mechanisms involved in horizontal gene transfer between environmental bacteria is the conjugative transfer by mobile elements including plasmids, transposons, integrons embedded on plasmids or transposons and insertion sequences (Zhang *et al.* 2009b).

The previous chapters clearly showed a prevalence of IS*CRs* in the biased hospital environment. However, the prevalence and route of dissemination of IS*CR*s in the community is not so clear and needed further investigation. Ria de Aveiro, Portugal and other environments geographically separated (Cardiff lakes) were investigated to determine whether location was a biased factor for the occurrence of IS*CR*s. To hypothesize on the route of dissemination, fish guts isolates were collected and screened for the presence of IS*CRs.* For this particular

purpose *Liza aurata* species was chosen based on its feeding behaviour, which is characterized by a regular contact with the sediment being often extensive to the whole water column.

7.2 Results and Discussion

7.2.1 Screening of IS*CR* in bacteria collected from fish guts

It has been recognized that MDR commensal bacteria inhabiting animals gut are an important source of bacteria causing opportunistic infections. Also, these bacteria can act as reservoirs of antibiotic resistance gene being a source to spread bacteria infecting humans (Hawkey and Jones 2009). Based on this hypothesis, 100 Gram-negative isolates were recovered from guts of fish belonging to *L. aurata* species. Five animals were captured in each of three different sampling points, according to different sources and levels of contamination. The three sampling sites were located in the estuary Ria de Aveiro, a clean sampling point, used as reference site for environmental studies by other authors (Nogueira *et al.* 2010), a sampling point contaminated with waste from a slaughterhouse and a third sampling point contaminated with heavy metals, namely mercury (Figure 39). The isolates were recovered as described in Material and Methods section 2.1.

Hybridization experiments were conducted in order to evaluate the occurrence of insertion sequence common regions, namely IS*CR1* and IS*CR2*, among the population under study. Colony blot was employed to detect the presence of IS*CRs* using the methodology described in Material and Methods sections 2.7.1 and 2.7.3. An environmental origin for several antibiotic resistance genes as been hypothesized, for instance the CTX-M genes from *Kluyvera* genera (Rodriguez *et al.* 2004). Nevertheless in this study and among the population under study was not detected the presence of IS*CRs*. These results can be explained either due to the fact that a small number of animals was analysed or to the low pressure in these sampling points, to select for structures such IS*CRs*. Toleman and co-workers hypothesize on the transfer of IS*CRs* from the environment to the hospital

settings (Toleman and Walsh 2008). Moreover, other reports show the presence of IS*CRs* in isolates collected from companion animals or food, therefore the boundaries of genes transfer are becoming narrower (Ma *et al.* 2009). Meunier and colleagues detected the occurrence of IS*CR2* in *E. coli* isolates collected from diseased animals from France (Meunier *et al.*). Also, the occurrence of resistance genes, such as *bla* and *sul* genes, among isolates collected from wild animals has been described in Portugal (Costa *et al.* 2006; Poeta *et al.* 2009). In isolates collected within the hospital environment, those genes are often found associated with the IS*CRs*. Therefore it might be suggested that this association is likely to be found also in isolates collected from wild animals.

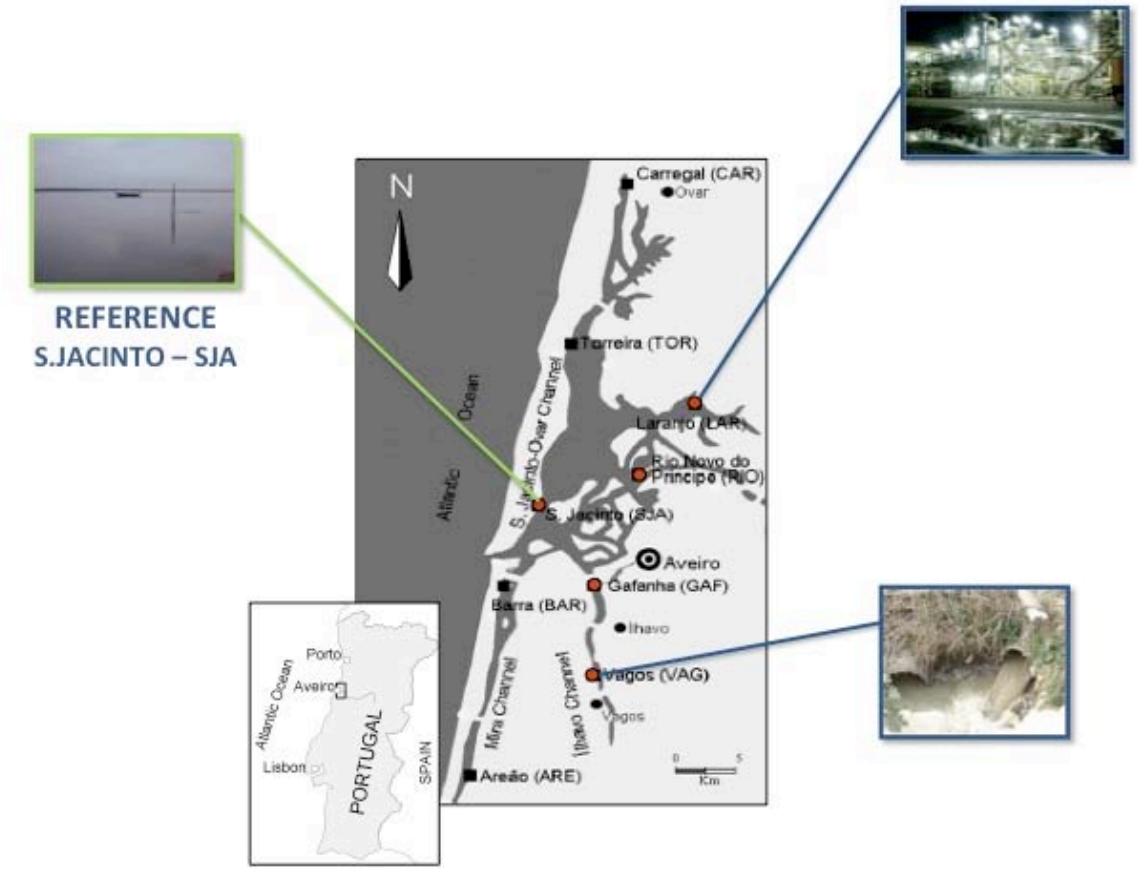


Figure 34 – Geographic location of sites where the fish included in this studied were captured.

7.2.2 Screening of IS*CR* in bacteria collected from water samples

In surface water, it is difficult to find an area where antibiotics cannot be detected, except for the pristine site in the mountains before the rivers or streams going through urban or agricultural areas (Hawkey and Jones 2009). One hundred *E. coli* isolates collected from water lakes around Cardiff, Wales (United Kingdom) were screened for the presence of IS*CRs.* Colony blot was employed to detect the presence of IS*CRs* using the methodology described in Material and Methods sections 2.7.1 and 2.7.3. However, it was not detected the presence of IS*CRs* among these isolates. Gram-negative bacteria, where a wide range of mechanisms of resistance is encoded by a plethora of genes, many of which are highly mobile, may pose substantial problems (Hawkey and Jones 2009). Therefore, 750 Gram-negative isolates were collected from the water column in three sampling sites in the estuary Ria de Aveiro, which presented different levels and sources of contamination, namely: Costa Nova, Cais do Chegado and Cais do Sporting. Subsequently, the same methodology was employed to screen for the IS*CR1* and 2 elements. The presence of IS*CR*s was not detected. Nonetheless, water constitutes not only a way of dissemination of antibiotic resistant organisms among human and animal populations, as drinking water is produced from surface water, but it is also the route through which resistance genes are introduced in natural bacterial ecosystems. In such systems, nonpathogenic bacteria could serve as a reservoir of resistance genes and platforms (Baquero *et al.* 2008b). The results obtained suggest that IS*CRs* may be rare in the environments studied. However, given the global panorama of dispersion of the mobile genetic elements, one can predict that it is a matter of time to find these structures within these environments. Nevertheless IS*CRs* free locations should be preserved and efforts should be put in surveillance studies for those genetic elements as well as other resistance genes. Despite the fact that no evidence was found of the occurrence of IS*CRs* among these isolates, it is likely that they will become present in a few years, facilitating the movement of resistance determinants.

7.2.3 General Conclusion

Based on the results obtained, the geographically separated environments did not seem to be a biased factor for the occurrence of IS*CRs.* Moreover, IS*CR* could not be detected in isolates collected from the selected sampling points.

It was not possible to hypothesize on the route of dissemination of the IS*CRs* since their presence could not be detected in the isolates collected from the fish guts. However, this type of study should be considered preliminary and should be repeated including a larger number of animals as well as different species.

8. General Discussion

8.1 IS*CRs*

The occurrence and the genetic context of IS*CR* elements in clinical and environmental isolates were investigated during this study. IS*CRs* are responsible for the mobilization of antibiotic resistance genes, including the *bla*$_{CTX-M}$ genes, from any location, by a rolling-circle transposition mechanism, allowing genetic rearrangements by homologous recombination into a class 1 integron (Toleman *et al.* 2006a)

The genes encoding quinolone resistance (*qnr* genes) are typical examples of complex integrons in which IS*CR*s have played a major role in bringing together antibiotic resistance genes combinations, including fluoroquinolone and extended-spectrum β-lactam resistance genes, on single conjugative plasmids.

IS*CR1* is always found as part of a complex class 1 integron (Toleman *et al.* 2006a) and this feature was fully investigated in this study.

In *K. pneumoniae* and *C. freundii* isolates the genetic context of these structures was well characterized revealing that in both cases it was associated with class 1 integron and with a *qnr*A gene. Since the first description of the “common regions” in the early 1990s, several reports worldwide describe this association, highlighting the success in their dissemination as well as in the movement of antibiotic resistance genes among different species. (Garnier *et al.* 2006; Quiroga *et al.* 2007; Su *et al.* 2008).

In contrast, IS*CR2* has never been found associated with class 1 integrons. IS*CR2* is intimately associated with antimicrobial resistance genes especially sulphonamide resistance (*sul*2) and it has been found located on a number of plasmids adjacent to chloramphenicol/florphenicol resistance gene, *flo*R, and *sul*2 gene (Toleman *et al.* 2007a). However, in the Portuguese *A. baumannii* isolates was found to be chromosomally located, despite the fact that the isolates carried large plasmids. Nonetheless, the study of the genetic context of this IS*CR2* element revealed that it was associated with the *sul*2 gene reinforcing the association between IS*CR* and the sulphonamide resistance. The comparative study revealed that in the Argentinean isolates, IS*CR2* was found to be associated

with the *flo*R gene and therefore in a different genetic context than that from the Portuguese population. A possible explanation for that association is the fact that florphenicol it is widely used in the veterinary medicine in Latin America, specially in Brasil and Argentina. In these countries meat exportation has a major impact in the economy, which may be reflected in the over-use of antibiotics and ultimately into the emergence of resistance. The association of resistance genes, such as *flo*R, to structures that can provide their dissemination among bacteria ensuring its "survival", is just a matter of time. In Europe, florphenicol was licensed for the control of bacterial respiratory tract infections in cattle and pigs in 1995 and 2000, respectively, and is active against chloramphenicol resistant isolates (Blickwede and Schwarz 2004). Therefore, it is possible that the selection of this particular association still unnoticed.

8.2 Class 1 integrons

Class 1 integrons containing IS*CR* elements are known as complex class 1 integrons. They contain the classic integron structure and a second copy of the 3'CS. Between the two copies of the 3'CS is a 2.1 kb IS*CR*, that has been identified in In*6*, In*7*, In*117*, In*34*, In*35*, In*37* and In*601* followed by a variable region that contains resistance genes, e.g. *dfrA10*, *catII*, bla_{DHA-1} (*pSAL-1*), $bla_{CTX-M-9}$ or $bla_{CTX-M-2}$, (Su *et al.* 2008). In the present study, complex class 1 integrons were found mainly in *K. pneumoniae* and *C. freundii* isolates, for instance, the In*37* which has been previously described in countries such as China and Argentina (Quiroga *et al.* 2007).

The gene cassettes arrays found were already described elsewhere either in different species or different backgrounds demonstrating the dissemination of these structures worldwide and between species. Additionally, a linkage between integron carriage and the resistance exhibited by the isolates to some antibiotics such as aminoglycosides, trimethoprim and β-lactams can be established. In all the species studied and also in the gene arrays found *aadA* and *dfr* variants were predominant.

Although nowadays streptomycin and spectomycin antibiotics are rarely used in patients clinical treatment, *aadA* gene cassette variants remain prevalent in a high number of arrays among Gram-negative species. White and colleagues (2001) reported that might suggest that these genes are not easily excised from the variable region. Also, an antibiotic may no longer be frequently used in therapy, but this does not necessarily mean that gene(s) coding for its resistance are going to be lost. However, several studies have shown that the exposure to a new antibiotic leads to a repositioning of the gene cassettes by the integrase. The closest gene to the promoter will be the more efficiently expressed (Collis and Hall 1995). As resistance genes can persist within elements such as integrons, the association of the latter with IS*CR* elements exponentially potetiates their dissemination. Surveillance studies of this association are crucial, especially within the hospital environment, since the association of these two elements can pose a major risk for the patients by contributing for therapeutic failure.

8.3 β-lactamase genes content

The clinical implications of β-lactamases carriage, especially ESBLs, are numerous and can compromise patient's treatment. Thus, rapid detection of ESBL-producing strains is essential to the implementation of the appropriate therapy and to monitor the development of resistance (Paterson and Bonomo 2005). In the present study, different types of β-lactamases enzymes were detected, namely narrow-spectrum β-lactamases, ESBLs, carbapenemases and broad range β-lactamases. Narrow-spectrum β-lactamases, represented by bla_{TEM}, were the most prevalent genes found. PCR and subsequent nucleotide sequence analysis performed in the clinical isolates, identified bla_{TEM-1} in virtually all species with the exception of *A. baumannii.* As expected, bla_{TEM-1} was the predominant *bla* gene among the isolates. $bla_{TEM-1/2}$ have been shown to be widely disseminated among Gram - negative bacilli and are the main resistance mechanism to narrow spectrum cephalosporins (Schmitt *et al.* 2007). Moreover, carbapenemases represented by oxacilinases hydrolyzing carbapenems, namely bla_{oxa-40} were only

detected in some *A. baumannii* isolates. In contrast, ESBLs were detected in all the species and in a high percentage. This fact has been frequently observed in the recent years by several authors and it represents a serious concern since ESBLs inactivate the majority of the β-lactams frequently used such as penicillins and first to third generation cephalosporins (Poirel *et al.* 2008). Among the ESBLs detected bla_{CTX-M} genes were the most frequently found and with a higher incidence in *E. coli* isolates. Interestingly those genes were not detected in *K. pneumoniae* strains resistant to third generation cephalosporins, where their hidrolysing activity was replaced by the presence of broad range β-lactamases, namely $bla_{DHA-1.}$ bla_{CTX-M} are usually plasmid located and have a preferential hydrolytic activity against third generation cephalosporins (Eckert *et al.* 2006). The rapid dissemination of bla_{CTX-M} worldwide and among different species is a major threat to clinical efficacy of third generation cephalosporins treatment (Munday *et al.* 2004). β-lactamases encoding genes are usually associated with insertion sequences (ISs), specially in Enterobacteriaceae and *P. aeruginosa* strains. In 2003, the association of a particular insertion sequence, IS*Ecp1,* with the $bla_{CTX-M-15}$ was reported and it has been identified in several Enterobacteriaceae isolates (Poirel *et al.* 2003). Subsequently, several studies confirmed the presence of this element upstream of the bla_{CTX-M} gene. In the present study, $bla_{CTX-M-15}$ when present was always associated with the insertion sequence IS*Ecp1*, reinforcing the linkage between these two structures.

Overall, the results of this study show that, as expected given the high selective pressure of the clinical environments, the emergence and prevalence of β-lactamases is high and appears to be disseminated among different nosocomial species that become MDR and are able to cause severe infections. Thus, surveillance studies either in MDR bacteria or bacteria exhibiting low level resistance are of utmost importance. The information provided constitutes the basis of epidemiological data and may contribute to the implementation of new measures within the hospital environment.

8.4 *qnr* genes

Different transferable *qnr* genes have been described so far, where *qnr*A, *qnr*B and *qnr*S appear to be the most common variants (Jacoby *et al.* 2008). In the present study, only *qnr*A and *qnr*B genes were detected, and both were found in *K. pneumoniae* and *C. freundii* isolates. *qnr*S was not detected among the isolates screened which leads to suggest that the former variants are dominant in our hospital. In both *K. pneumoniae* and *C. freundii*, *qnr*A genes were found associated with IS*CR1*, that is located on a plasmid and embedded in a complex *sul1*-type integron. *qnr*B variants were not associated with neither the *orf1005* gene nor the IS*CR1* recombinase gene. Nonetheless, hybridisation experiments revealed that two *K. pneumoniae* and the *C. freundii* CIT1 harboured *qnr*A and *qnr*B genes simultaneously and both of them were located on large plasmids. Attempts to transfer by conjugation, the *qnr* genes located on those plasmids were unsuccessful in both *K. pneumoniae* and *C. freundii*. Moreover, isolates from both species carried those *qnr* genes on plasmids with similar sizes suggesting that the size of the plasmid may be the limiting condition of this transfer *in vitro*. Interestingly, plasmids with similar sizes harbouring both *qnr*A and *qnr*B genes simultaneously, were found in both *K. pneumoniae* and *C. freundii*, collected in the same hospital. Based on these findings it may be suggested that in the laboratory it might be difficult to accurately reproduce the appropriate conditions to the horizontal gene transfer *in vitro*. However, that does not necessarily mean that within the hospital environment and under favorable conditions, the gene transfer does not occur. Although clinical implications of the *qnr* genes are still unclear, it is accepted that these are responsible for low-level resistance to fluoroquinolone resistance (Yang *et al.* 2009). Thus the spread of such resistance determinants is concerning, since it may play a significant role in the emergence of resistant mutants and consequently therapeutic failure. Moreover, the epidemiology of complex *sul1*-type integrons, as well as the association of *qnr* genes to IS*CR1* in the bacterial population analysed highlights the need for surveillance that helps to predict the prevalence of these mechanisms.

8.5 Environmental screening

Antibiotic resistant organisms enter into water environments from human and animal sources (Baquero *et al.* 2008a). Evidence of the role played by wild animals, wastewaters and estuarine reservoirs in antibiotic resistance maintenance and dissemination has been provided by several reports (Costa *et al.* 2006; Henriques *et al.* 2008; Moura *et al.* ; Moura *et al.* 2007). Bacteria found in these environments are able to spread their genes into water-indigenous microorganisms, which may in turn contain other resistance genes. The resistance is maintained by many antibiotics from industrial origin that circulate in water environments, potentially altering microbial ecosystems (Baquero *et al.* 2008a). A policy for preventing mixing human-originated and animal-originated bacteria with environmental organisms seems advisable, but difficult to implement.

Despite the fact that IS*CRs* could not be detected in the environmental bacteria analysed in the present study, regardless the origin of the isolates, should not rush us to conclude that these are not present in these environments. Other authors report the presence of resistance genes, often found associated with IS*CR,* in the same sampling points (Henriques *et al.* 2006; Henriques *et al.* 2008; Moura *et al.* 2007) therefore one can suggest that a wider screen would probably give positive results.

8.6 Conclusions

The results that are included in this thesis are of utmost importance, since they constitute the first study carried out in a Portuguese hospital providing therefore a realistic panorama of the dissemination of the IS*CR* elements within this highly selective environment. Additionally, this study also helped to highlight the importance of the association and implication of these elements in the dissemination of antibiotic resistance genes within hospital environment.

Thus, the following major conclusions can be withdrawn:

1) A biased occurrence of IS*CR*s in environments where the antibiotic pressure is stronger, namely the hospital environment is observed.

2) The screening of mobile genetic elements such as IS*CR* elements and *Tn*5090 transposons in the clinical isolates showed a high occurrence of those elements, in different genetic contexts and associated with different antibiotic resistance genes.

3) In the *A. baumannii* isolates the association of the IS*CR2* with the *sul2* gene does not have a strong impact in the resistance profile of these bacteria. However, in *E. coli*, *K. pneumoniae* and *C. freundii* IS*CR1* was found associated with complex class 1 integrons, that harboured different antibiotic resistance genes encoding resistance to different classes of antibiotics. Since these genes are located in mobile elements it may therefore enhance their dissemination.

4) Since ISCR were found intrinsically linked to antibiotic resistance genes and can mobilize the transfer these resistance determinants, a continuous surveillance of these elements is crucial to ensure successful treatments.

5) The results obtained in this study highlight that precautions should be taken to prevent the dissemination of these resistance determinants within the hospital environment. The occurrence of MDR populations within this biased environment requires the implementation of simple measures (such as reduction of beds per ward) that combined with periodic surveillance control, will certainly contribute to avoid the dissemination of these bacteria among hospitalized patients.

6) Although IS*CR* elements could not detected in isolates collected in the natural environment, wither surveillance studies should be performed. These studies would be essential, specially in areas close to the hospital discharges, aquacultures and domestic effluents.

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